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Determining the efficacy of potassium oxalate containing strips to reduce dentinal fluid flow *in situ*

by

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A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Master of Science in the Faculty of Health Sciences.

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Abstract

The consensus stands that dentine hypersensitivity is caused by stimulus applied to exposed dentinal tubules inducing movement of fluid through the tubules and subsequent activation of mechanoreceptors and correlated pain sensation.

The study aims were to test the ability of three solutions to remove any smear layer and leave patent dentine tubules following sectioning. To establish a standard sample brushing protocol for stabilising hydraulic conductance of dentine. To develop a flow cell model to measure the efficacy of a gel strip containing 3.14% potassium oxalate to occlude dentinal tubules as well as adapting the model to include an in situ phase. Finally, to corroborate any findings with dentine micrographs.

Hydrodynamic flow rates were measured at baseline and post 10 minute treatment, some discs were left untreated. One treated and one un-treated disc were housed in palatal appliances worn by 20 participants for consecutive 14 days. Following the in situ phase, flow rates were re-measured and dentine discs were imaged using FIB-SEM and EDX before being fractured and imaged with SEM.

Treated discs showed a reduction in flow rate from 90.3µl/min at baseline to 36.7µl/min following treatment, which was highly significant ($p<0.0001$). Following oral housing the flow rate was further reduced to 9.6µl/min. The flow rate of the non-treated discs reduced from 123.8µl/min at baseline to 40.0µl/min after oral housing, also highly significant ($p<0.0001$). Flow rate comparisons between treated and non-treated discs following housing in the oral environment showed that treated discs provided a significantly greater reduction in flow rate compared to non-treated discs ($p=0.0038$). Micrographs allowed identification of crystalline blockages within the dentine tubules of treated samples, supporting the results from the flow cell.

This model was effective in showing the immediate occluding potential of the treatment whilst also showing its resilience following 14 days exposure to the oral environment.

Dedication and Acknowledgements

I would like to dedicate this work to my parents Paul and Alison Chapman whose hard work and dedication to our family have proved inspirational and encouraged me to progress.

I declare my sincere gratitude to Professor Nicola West who provided me with an incredible opportunity to develop as a researcher. The enthusiasm and diligence you present every day is truly admirable.

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Finally, I would like to thank Dr Maria Davies, Dr Priya Bahal and Dr Natalie Wood who have each provided a spark with which to endeavour.

Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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List of Abbreviations

CEJ	Cementoenamel Junction
CSPS	Calcium sodium phosphosilicate
DH	Dentine hypersensitivity
DI	Deionised (Water)
EDJ	Enamel-dentine junction
EDX	Energy dispersive X-ray spectroscopy
FIB-SEM	Focused ion beam scanning electron microscope
GPa	Gigapascal
HS	Hartmann’s solution (Saline)
ITD	Intertubular dentine
keV	Electronvolt
NCCLs	Non-carious cervical lesions
PTD	Peritubular dentine
QoL	Quality of life
RCTs	Randomised clinical trials
SEM	Scanning electron microscope
VAS	Visual analogue scale
VRS	Verbal rating scale

1 Introduction

1.1 Dentine Hypersensitivity

1.1.1 Presenting signs, symptoms and quality of life

Dentine Hypersensitivity (DH) is a painful oral condition prevalent throughout the world. A consensus on the definition has defined it as "pain arising from exposed dentine in response to stimuli, typically thermal, evaporative, tactile, osmotic or chemical and which cannot be ascribed to any other form of dental defect or pathology" (Addy and Dowell, 1983). The pain is transient and characterised by its brief and shooting nature with an instantaneous response felt (Trowbridge, 1985).

The most commonly cited sources of evoking the pain associated with DH are cold foods and drinks or cold air, either through consumption of cold foodstuffs or the evaporative effect when inhaling air, particularly on a cold day. Pain may also be stimulated when eating foods that are hot, sweet or sour but often to a lesser extent (Orchardson et al., 1994). Physical stimulation of dentine such as the pressure exerted by forceful tooth brushing may also be a source of DH pain if the surface is compressed (Pashley, 1990). The pain arising from DH can be significant enough to affect a patient's quality of life (Bekes and Hirsch, 2013).

Quality of life (QoL) concerns have historically been regarded as secondary to clinical outcomes when evaluating if a treatment has been a success. Increasingly however, attention is paid to the patient's own thoughts and feelings regarding treatment options and the impact an oral condition may have on their life (Bekes and Hirsch, 2013). The U.S Surgeon General's report on oral health defines QoL considerations as "a multidimensional construct that reflects (among other things) people's comfort when eating, sleeping and engaging in social interaction; their self-esteem and their satisfaction with respect to their oral health" (Bekes and Hirsch, 2013).

A diverse range of studies worldwide have examined the effects of DH on QoL. In a study of patients who attended a general dental practice and reported having sensitive teeth 34% perceived cold stimulus to be the cause of most discomfort. This resulted in being unable to drink cold water or eat ice cream without pain. In addition, 14.1% of these attendees experienced similar discomfort when their teeth were cleaned by a dentist or hygienist (Gillam et al., 1999), which could lead to reduced dental appointment attendance and future dental issues. Further evidence regarding the impact of DH on QoL was obtained more recently in a study of young adults in Europe where 28.4% reported that their DH was either important, or very important to them (West et al., 2013b). Furthermore, a study of Brazilians between the ages of 35-59 reported that 54.5% experienced at least one QoL issue relating to sensitivity that negatively affected them either 'fairly often' or 'very often' (Wagner et al., 2016). Where patients are experiencing pain to the extent that it impedes their ability to eat, drink, brush their teeth and even breathe they generally respond by changing their behaviours to avoid certain food and drinks resulting in an impact on their QoL and eventually necessitating self or professional treatment (Bekes et al., 2009).

Care must be taken when diagnosing DH as pain in the oral cavity can often present in a similar manner to the patient whilst originating from a number of different conditions. These include caries, chipped teeth, fractured restorations, a response to restorative treatment and leakage around restorations, cracked tooth syndrome, restorations left in traumatic occlusion, palatogingival groove, vital bleaching and atypical facial pain (Addy, 2002). Therefore, any diagnosis of DH will need to be reached via the exclusion of any periodontal or dental conditions that may also cause pain (Trushkowsky and Oquendo, 2011). The criteria described in the definition of DH provides the basis of any diagnosis. The outer dentine is exposed due to recession of the gingiva and the cementum layer removed or the loss of enamel. The dentinal tubules must also be patent if stimulation of the surface is to occur (West, 2008). Screening for DH requires the clinician to consider a multitude of factors. These include identifying any predisposing factors in relation to the patient's oral hygiene and their dietary routine, which could result from tooth wear, abrasion, erosion, attrition or any combination of these factors. It is necessary to rule out symptoms with a similar pain as caused by DH, if any other condition is present it must be treated prior to any diagnosis of DH being made (Canadian-Advisory-Board, 2003)

Where a clinician has excluded all alternative pathologies through differential diagnosis an examination for signs of DH can be performed. It could be suggested to perform tactile stimulation with a probe in the mesiodistal (or distomesial) direction across the exposed dentine (Gillam, 2013). In addition, the Schiff Cold Air scale is commonly employed measuring the pain response from a cold air-blast (Schiff et al., 1994) and the clinician assigning a score on a 3-point scale in relation to the patient's reaction.

Where these tests are to be performed as part of a clinical trial, quantifiable data can be recorded by requesting the patient to indicate the level of pain felt with the use of a visual analogue scale (VAS). The patient is asked to place a mark on a 100 mm line to indicate the level of pain experienced from no pain to worst pain ever experienced (Gillam, 2013). This scale can be found to correlate with the intensity of pain established by alternative methods (Jensen et al., 1986). The wide range in response data allows for a measured response to sensitive changes in pain intensity and the data collected permits statistical analysis (Briggs and Closs, 1999). However, some limitations can be found, some patients find translating the sensory experience into a linear representation difficult to grasp (Wewers and Lowe, 1990) and each patient may interpret the scale differently (McGuire, 1984).

A verbal rating scale (VRS) can be used as an alternative and patients, particularly the elderly, seem to find them easier to use (Kremer et al., 1981, McGuire, 1984). A list of adjectives describing different levels of pain is provided to choose from with the method attaining high compliance rates (Jensen and Karoly, 1992). The results provide simple ranked data with a fixed number of responses, therefore the differences between each category cannot be assumed to be equal, suggesting that VAS scores more accurately record treatment effects (Briggs and Closs, 1999). Despite any limitations both techniques place minimal demands on the patient and can be easily employed in clinical and research settings.

To supplement any clinical findings a patient may be asked to record details of their food and drink intake in the form of a daily diary to help both the clinician and patient identify erosive elements that could contribute towards the onset of DH (Orchardson and Gillam, 2006).

Oral sensitivity experienced by a patient can be as a result of any number of conditions. The term DH has been interchanged with dentine sensitivity, pulpal sensitivity, tooth sensitivity, cervical sensitivity, tooth hypersensitivity and root sensitivity to describe similar types of sensitivity (Von Troil et al., 2002). However, the definition of DH as stated by Addy and Dowell specifically states that a diagnosis of DH relies on the condition “not being ascribed to any other form of dental defect or pathology” (Addy and Dowell, 1983). The site location and participant selection for clinical studies examining DH leads to a significant distortion in prevalence rates for the condition. For example, in a study of patients referred to a specialist periodontology department, 84% were recorded as having symptoms of DH. The authors suggested this was as a result of recession due to periodontal disease or to periodontal treatment such as root planning (Chabanski et al., 1997). This study would therefore not fit the definition of DH as an alternative pathology is stated.

The sensitivity experienced post periodontal surgery may occur as a result of gingival recession and dentine exposure but is considered a separate condition from DH. Furthermore, sensitivity does not always follow periodontal therapy. In a study of 11 patients conducted at the Department of Periodontology at Lund University, Sweden, in 1991 only 3 of the patients experienced a significant increase in probe and air sensitivity following supragingival scaling, increasing to 6 patients after subgingival scaling. Of these 6 patients, 5 stated that their sensitivity increased further in the week after the treatment (Fischer et al., 1991). The finding that pain increases soon after treatment concurs with studies performed in vitro where it has been shown that root surfaces treated by root planning or burring are initially concealed by a smear layer of microcrystalline debris of cementum and dentine (Addy et al., 1987a). This smear layer occludes the newly ground surfaces resulting in teeth less sensitive to stimulation for up to one week (Brannstrom, 1965), however, in vivo, this protective layer is gradually lost due to the effects of dietary acids and tooth bushing (Addy et al., 1987a). Interestingly though, unlike true DH, sensitivity following periodontal treatment is short lived, and has been shown to return to pre-treatment levels after 4 weeks (Tammaro et al., 2000).

Another type of sensitivity that can be confused with DH is that caused by vital tooth bleaching. The application of bleaching agents such as hydrogen peroxide or carbamide peroxide have grown in popularity as people pursue an aesthetic improvement in the colour shade of their enamel. Evidence suggests that these tooth whitening procedures may negatively affect the hard tissues by increasing the porosity of the enamel structure causing loss of protein concentration and demineralisation, as well as penetration into the pulp (Bowles and Ugwuneri, 1987) but the effect tends to be mild and temporary. One study concluded that sensitivity occurred primarily in the first 2 weeks of treatment and had decreased by the final 4th week, although where gingival recession was recorded participants continued to report sensitivity in the 4th week (Jorgensen and Carroll, 2002). This suggests a positive correlation between gingival recession and reported sensitivity though the pathology here is sensitivity caused by penetration of peroxide into the pulp and is therefore not considered DH. However the treatment for this sensitivity can be similar, for example the use of a toothpaste containing potassium nitrate for 2 weeks before and during the bleaching cycle could reduce or remove the sensitivity by desensitising the nerves and occluding tubules (Pashley, 2008).

Prevalence rates of DH will only be considered true DH where a study has taken satisfactory steps to rule out conditions such as periodontal disease and sensitivity as a result of vital tooth bleaching. These pathologies can be considered separate to conditions such as gingival recession and the formation of non-carious cervical lesions (NCCLs), which have been suggested to be multifactorial conditions developing in part due to overzealous tooth brushing enhanced by an acidic environment (Heasman et al., 2015).

1.1.2 Prevalence

The prevalence of DH throughout the world has been reported with extremes in the range of figures quoted due to the variety in the data collection techniques and the diversity of the cohorts studied with figures ranging from 1.34 – 98% (Chabanski et al., 1997, Bamise et al., 2007). To obtain prevalence figures that more accurately reflect DH in the population, larger studies which are more inclusive and capture participants from a range of backgrounds and countries are necessary. A recent study of 3187 adults aged 18-35, recruited across 7 European countries, was conducted through the use of a self-administered questionnaire and clinical evaluation in response to a cold air stimulus. The investigation concluded with 42% of participants fulfilling the criteria for a diagnosis of DH (West et al., 2013b). By contrast a UK study of 3593 patients with ages ranging from 15 to 79 years who were assessed when attending 1 of 12 participating general dental practitioners over the course of 1 month, prevalence of DH was found to be far lower at 3.8% (Rees, 2000). This study did not employ a questionnaire and relied upon a verbal confirmation of sensitivity by the patient, with only those who indicated they had sensitive teeth receiving a clinical assessment in which sensitivity was confirmed following a cold air blast stimulus. The differences in prevalence rates between these 2 studies may reflect differences in the ages of the participants recruited, and the reliance of the latter study on potential participants confirming they had sensitivity before a clinical exam was undertaken.

Diagnosis of the symptoms of DH are predominantly made between the ages of 15 to 70 years with the peak found between 20 to 40 years, these findings are often mirrored by greater levels of gingival recession found in people between the ages of 30-40 years (Pashley, 2008). The decrease in levels of DH found in older patients is reflected by a reduction in the permeability of dentine found in older teeth. The hydraulic conductance of dentine from a group of patients between 45-69 years of age was found to be 80% less than dentine from those in a 20-28 years age bracket, which was thought to be as a result of increased intratubular crystals found in the aged dentine (Tagami et al., 1992).

Studies in less well-developed countries have also been undertaken. A 2016 study in India was conducted using both a verbal rating scale and questionnaire to quantify the patient's own perceptions of DH before moving on to a clinical evaluation. 404 patients were assessed firstly with a scratch on the tooth surface performed with a dental instrument. This was followed by a 10-minute wait before the reaction to a cold air blast was scored. 20.6% of the participants subjected to these tests were confirmed to have hypersensitive dentine (Haneet and Vandana, 2016). A study of patients attending a dental clinic in the city of Rio de Janeiro, Brazil requested that patients self-report DH via a questionnaire and out of a total of 635 subjects screened 25% stated they suffered from the condition. However, when subjected to clinical tests involving cold air blasts and tactile feedback from scratching with a probe the pervasiveness of DH was found to be 17% (Fischer et al., 1992).

The variations in prevalence rates for DH can be explained by the survey methods used, the population studied and the socio-economic condition of the region or country under investigation. Diversity in national or regional economic development, a populations diet, oral hygiene standards and attitudes towards oral disease will all contribute to the different prevalence rates documented for DH (Walters, 2005). In countries where gingival recession is on the increase the numbers of people suffering with DH are likely to follow. This can be as a result of excessive or forceful tooth brushing particularly when combined with a modern acid containing diet (Pashley, 2008).

Prevalence studies investigating numbers of people with a diagnosis of DH often report a divergence in the distribution of sufferers between genders. The number of females affected is frequently found to be greater than males participating in the same study, however the level of statistical significance varies.

An American study of 787 adults attending general dental practices employed both a questionnaire discussion with the patient as well as clinical cold air blast tests to confirm the numbers of patients with DH of which 57.8% were female and 42.2% male (Cunha-Cruz et al., 2013). A similar split along gender lines was found in a Chinese study that employed the same diagnostic techniques on 2120 adult participants. Of the 804 cases where DH was diagnosed, 59.5% were females and 40.5% were males resulting in a statistically significant male to female ratio of 1:1.5 (Ye et al., 2012). An even higher male to female ratio of 1:2.5 was established in a British study of 4841 participants using the cold air blast technique to confirm DH (Rees and Addy, 2002).

While there is an established trend for higher levels of DH found in females the results of some studies do not provide a significant split between genders, perhaps suggesting that the divergence could be due to an overrepresentation of females in study data. In one of the earlier investigations of DH prevalence a Scottish sample population of 369 participants reported a male to female ratio of 1:1.33 which was not a statistically significant result (Flynn et al., 1985). This result was shared by a more recent study with data collected from 284 Australian dental practitioners investigating a total of 12,692 patients finding that the mean extent of DH in women was not statistically different from that of men (Amarasena et al., 2011).

There are several possible reasons for the apparent higher prevalence of DH in females and they are intrinsically linked to established dental science. It is noted that the habit of tooth brushing seems to be undoubtedly correlated with exposure of dentine and is likely more a cause of gingival recession than loss of enamel (Sangnes, 1976). Epidemiological data proposes that the same sites in the mouth that are brushed well (Alexander, 1971) will result in the greatest prevalence of DH (Orchardson and Collins, 1987). It is also important to consider that in patients with DH, the affected sites in the mouth have a negative correlation with plaque scores (Addy et al., 1987b). These findings therefore suggest that tooth brushing is associated with DH.

The oral hygiene of females is acknowledged to be better than that of males from a young age (Addy et al., 1990) and the brushing style has been found to be more intensive (Sakalauskiene et al., 2011). The situation could be further exacerbated by females consuming more fruit containing natural acids leading to increased levels of erosion (Westenhoefer, 2005). This mixture of abrasion and erosion creates an optimal scenario to increase the etiological risk factors for DH.

The perception and reporting of pain may also provide a basis for discrepancies in the prevalence of DH between genders. Researchers studying pain have found that females are more likely than males to report a variety of temporary and persistent pains and to report more severe pain, more frequent pain and pain of longer duration than males (Unruh, 1996). This disparity in the reporting of pain may be as a consequence of biological, cultural and psychological differences, divergent social role expectations, situational factors and an individual’s past history (Levine and De Simone, 1991). It is thought that males have been socialised to suppress outward signs of pain and therefore under-report levels of pain (Riley et al., 1998) which in the context of studies assessing DH via pain scoring may explain the lower prevalence for male participants.

Where studies into DH are conducted in dental practices there may be an overrepresentation of females due to more frequent visitation (Nuttall et al., 2001) leading to selection bias in the study if this is not accounted for. Studies that rely on the participants to communicate oral health problems may show an increase in female prevalence as it has been found that females articulate their health issues more readily than males (Noone and Stephens, 2008).

The wide range of prevalence figures found for DH is matched by the wide range of diagnostic techniques, study designs and populations studied. Where patients undergoing periodontal treatment are tested for DH, prevalence rises to a maximum of 98% (Chabanski et al., 1997). However as stated above this study notes that DH is a diagnosis of exclusion (Canadian-Advisory-Board, 2003) therefore conditions that mimic DH must be ruled out. Studies which exclusively rely on a questionnaire model are also open to interpretation as the patient's own perception is relied upon, opening up the possibility that pain is reported as DH where in fact it is due to any other pathology (Dowell et al., 1985). Prevalence rates in questionnaire studies have been found to report levels of DH as high as 57.2% (Irwin and McCusker, 1997).

Questionnaires have been widely used, with or without clinical examination. The main issue with the questionnaire approach is the lack of universal objective validation before being used as a scale of sample. Moreover, different studies use different selection of samples with no clear inclusion and exclusion criteria adopted by the study. Additionally, many questionnaires do not correlate the condition with its aetiological and predisposing factors, which can lead to an inaccurate relative conclusion. The characterisation of DH by questionnaire combined with clinical examination can improve the outcome results of the research (Taha, 2014).

Accordingly, where studies are conducted with clinical tests on patients attending general dental practice the prevalence for DH can be found to reach a peak in adults of 34.1% (Ye et al., 2012). There is still a wide range however as in some regions prevalence has been reported as low as 3.8% (Rees, 2000). When taken as a whole, but in the absence of investigations that do not fit the definition, the prevalence of DH is consistently reported to be around 15% of the populace (Addy, 2002).

1.2 Tooth Structure

It is essential to appreciate the characteristics of enamel, dentine and pulp to have a clear understanding of the role of tooth structure on dentine hypersensitivity.

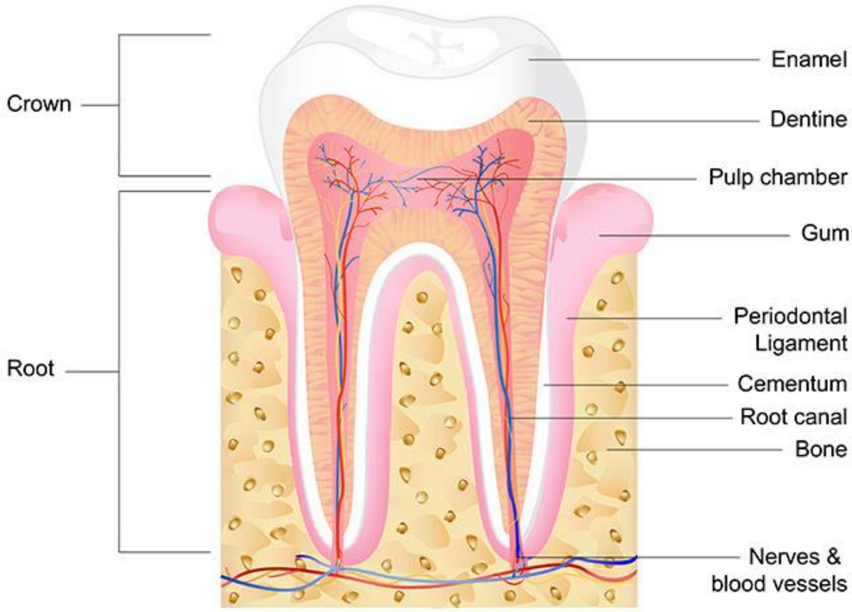


Figure 1.1 Schematic representation of tooth enamel, dentine and pulp (Giraldo, 2018)

1.2.1 Enamel

Enamel, the external surface of the tooth, is the hardest and most highly mineralized tissue of the human body. The position of enamel in a tooth is shown in figure 1.1. It is composed of inorganic calcium phosphate in the form of hydroxyapatite mineral crystals (96-97% by weight) surrounded by organic phase that is mainly proteins such as amelogenins and enamelins, carbohydrates, lipids, lactates and citrates (1%) and water (the remaining 2-3%) (Gwinnett, 1992).

Enamel provides a protective hard and wear resistant cover for the dentine and the pulp. This allows the tooth to withstand mastication forces throughout its lifetime (Nanci, 2014). The most critical area of enamel is the labial gingival third at the amelocemental junction. Studies have shown how the mean thickness of enamel at the gingival third tapers to less than 500 µm just below the healthy gingiva leaving this area as a vulnerable site for exposure of underlying dentine (Scott and Symons, 1974, Pahlevan et al., 2014, Davis and Winter, 1977).

Mature enamel is composed of an inorganic phase with a major mineral component of calcium, phosphate, fluoride, carbonate and magnesium. Sodium, chloride, strontium and lead are also present but in trace quantities among other elements (Akkus et al., 2016). Research has shown the mineral content varies substantially within enamel and that cervical enamel contains the least mineralisation. There is also a variation in the mineral content of enamel from the enamel-dentine junction (EDJ) to the outer surface of enamel where the outer surface of enamel contains the highest mineral content (Wilson and Beynon, 1989).

At the microstructure level, enamel is uniquely composed of mineral rich rods 3–6 µm in cross-sectional diameter as depicted below in figure 1.2. These rods are arranged with varying orientation and diameters throughout the enamel and imbedded in interrod substance (Braly et al., 2007).

The enamel rods are formed by the ameloblasts at the EDJ and extend to the outer enamel. Each rod is composed of millions of individual tightly packed hydroxyapatite crystallite with a lipid or protein coating (Addy et al., 2000). Rod-less enamel is known to be found in occlusal and cuspal-coronal enamel. This type of enamel is characterised by the hydroxyapatite crystallite being arranged perpendicular to the enamel surface and parallel to each other, resulting in elimination of organic rod boundaries. This absence of the organic material can result in a layer with higher mineral contents when compared to other parts of enamel (Robinson et al., 1971, Kodaka et al., 1991)

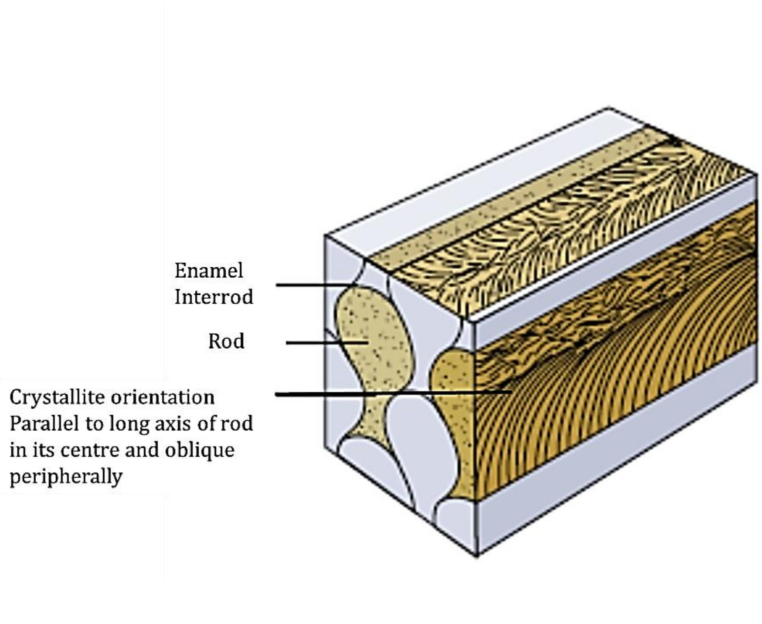


Figure 1.2 Schematic illustration of enamel's microstructure showing the enamel rods (Sakagushi and Powers, 2012)

1.2.2 Dentine

Dentine is a mineralised tissue that forms the bulk of the tooth; it is physically located between the exterior enamel layer and the interior pulp chamber as can be seen below in figure 1.3. It consists of 20% by weight organic matter (mainly type I collagen, present in 90% of contents and type V collagen present in 3% with the remainder made up of non-collagen proteins Phosphoproteins, Glycoproteins, Proteoglycans and lipids), 10% by weight water and 70% by weight carbonated hydroxyapatite. The hydroxyapatite crystals in dentine are smaller than those in enamel and larger than that of root cementum (Scully, 2002).

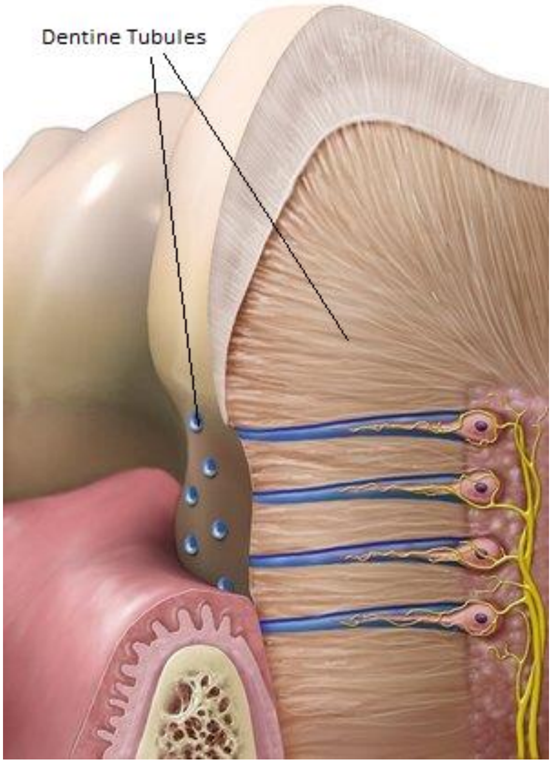


Figure 1.3 Schematic illustration of dentine inside the crown and at the EDJ (Sekijima, 2017)

Hardness of dentine ranges between 0.13 to 0.51 GPa while enamel hardness ranges between 3 to 6 GPa, indicating that dentine is much softer and more easily worn away. Furthermore, dentine is characterised by its compliant nature and low modulus of elasticity of between 18-24 GPa compared to that of enamel at 60-120 GPa. Dentine therefore serves as an elastic foundation for the hard, outermost enamel that absorbs stress inflicted onto the teeth. Dentine is very tough but enamel is much harder therefore they need to be joined together to provide a biomechanically compatible system. Due to dentine being a hydrated hard tissue it also acts as a protective enclosure for the pulp in close proximity (Sakagushi and Powers, 2012, Bechtle et al., 2010).

Dentine itself is a vital tissue, consisting of dentinal tubules, which are occupied by odontoblastic processes. Dentinal tubules, with a diameter of 1-2 µm, extend through the full thickness of dentine from the pulp to the EDJ following an S-curved course directed occlusally. These tubules are surrounded by hydroxyapatite crystals and the collagen matrix that is distributed in planes perpendicular to them. Dentinal tubules afford a degree of permeability to the dentine that can lead to the sensation of pain. This permeability plays an important role in response of the pulp to the oral environment since it permits movement of fluid through the dentine. The odontoblastic processes within dentinal tubules may extend through the dentine starting in close proximity to the pulp orientating towards the direction of the EDJ and are extensions of odontoblasts, which are the major cells involved in the formation of dentine (Scully, 2002, Scott and Symons, 1974, Orchardson and Cadden, 2001, Meyers et al., 2008).

The diameter and density of the tubules change along the thickness of dentine, as the distance from the pulp increases, the diameter decreases. This can be attributed to the fact that these tubules are not entirely single entities but branch laterally, in particular at their terminal ends at the EDJ (Scully, 2002). The number of tubules and radius increases from ~20,000 per mm^2 at the EDJ to ~45,000 per mm^2 towards the pulp (Pashley, 1996).

Dentinal tubules contain odontoblastic process, collagen fibres, mixture of proteins and are filled with dentinal fluid. The fluids within tubules have been suggested to play a role in relieving stresses directed toward the dentine through the enamel and periodontal ligament (Pashley, 1990). The dentine closest to the pulp tissue is the most sensitive and nerves penetrate the innermost layer of the dentine. (Orchardson and Cadden, 2001).

Dentine viewed via microscopy exhibits two clear formations. Firstly, peritubular dentine (PTD) forms ring like cross sections and occupies 3% relative area at the EDJ which increases to >60% at the mineralisation front where dentine forms (Takuma, 1960). It is made up of highly calcified or hyper mineralised tissue that “coats” the inside of the tubules (Miller et al., 1971). Secondly, the rest of the tissue surrounding the tubules is intertubular dentine

(ITD) and can be seen to be loosely arranged in comparison to the PTD. The content of organic matrix in the PTD is 1/3 that of the ITD but both are composed of the same hydroxyapatite and matrix proteins (Xu and Wang, 2012).

1.2.3 Pulp

The dental pulp is a highly specialised connective tissue that is surrounded by a rigid mineralised tissue which can be seen in figure 1.4 below. It is infiltrated by a network of vascular blood and lymph vessels with nerve bundles derived from the periodontal ligament emanating via the apical foramen of the root system. The pulp is uniquely characterised by the presence of odontoblast cells. Moreover, fibroblasts, defence cells like microphages, undifferentiated cells and intercellular matrix composed of collagen and ground substance (water, glycosaminoglycans and glycoproteins) are the main component of the pulp (Scully, 2002).

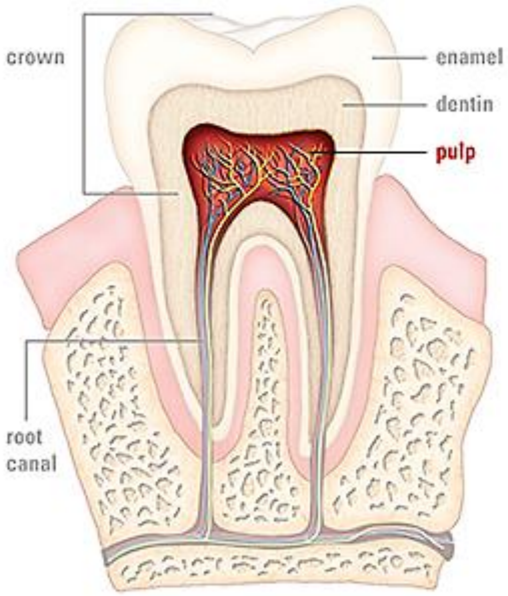


Figure 1.4 Schematic illustration of pulp inside the tooth (Mogram, 2010)

The pulp is well innervated, particularly beneath the odontoblast layer, and it presents a close relationship to the dentine through the odontoblastic process in the dentine tubules. The innermost dentine closest to the pulp is penetrated by nerves, although there is no evidence to suggest nerves span the full thickness of dentine (Scully, 2002).

Electrical impulses travel along axons or afferent nerve fibres which transmit signals from pulpal cells to synaptic terminals (Hildebrand et al., 1995). The axons can number in the thousands and mirror the distribution of the blood supply to the pulp. These bundles of nerves fan out to form the plexus of Raschkow where they end as free nerve endings that synapse onto the odontoblast cell layer (Bergenholtz et al., 2013). The pulp contains 2 types of sensory nerve fibres; myelinated A fibres (both A-delta and A-beta fibres) and unmyelinated C fibres. The A fibres, 90 percent of which are A-delta fibres, are situated both at the pulp-dentine border in the crown and concentrated in the pulpal horns which extend towards the roots. The C fibres are found in the pulp core and extend to a cell-free zone beneath the odontoblasts (Bender, 2000). Pain is transmitted by A fibres directly to the thalamus and presents as a fast, sharp pain which can be easily localised. The pain produced by C fibres is found to be slow, dull and aching in nature due to the numerous modulating interneurons before the thalamus is reached (Johnsen and Johns, 1978).

Pulp is integrally connected to dentine and as a result any physiologic and/or pathologic reactions in one of the tissues will also affect the other. The dentinal fluid forms around 22% of total volume of dentine and it plays a major role in the sensation connections between the pulp and dentine. It is an ultrafiltrate of blood from the pulp via dentinal tubules and forms a communication medium between the pulp along the odontoblastic layer and outer regions of the dentine (Orchardson and Cadden, 2001).

Damage to the dental pulp can be triggered by mechanical, chemical, thermal, and microbial irritants which can activate various types of inflammatory responses with the possibility of pulp tissue regeneration restricted. Whatever stimulus is applied the only sensation perceived is pain (Smith et al., 2008, Bjørndal and Mjör, 2001).

1.3 Causes of Dentine Hypersensitivity

1.3.1 Aetiology of dentine hypersensitivity

The aetiology of DH can rarely be defined as the result of a single factor, it is most often a multifactorial condition (West et al., 2014). The occurrence of DH is synonymous with exposure of the dentine surface to the oral environment. Foremost, two processes need to occur for DH to arise: the dentine surface of a tooth must be exposed (lesion localisation) and multiple dentinal tubules in close proximity to each other need to be open and patent to the pulp (lesion initiation). Dentine is exposed by either the loss of enamel or cementum. The loss of enamel can occur due to acidic erosion, abrasion and attrition. These chemical and physical processes interact together to wear enamel and expose dentine. The exposure of the dentine to the oral environment can also be attributed to the loss of cementum following gingival recession. Cementum can also be removed by abrasive action which will result in dentine exposure (Addy, 2002, Hirschfeld, 1939). Not all individuals who show signs of exposed dentine exhibit symptoms of DH, and this may be because the tubules might not be patent to the pulp. Indeed, exposed dentine does not always lead to DH, which may be attributed to the fact that not all factors which expose dentine causes dentinal tubules to become patent (Absi et al., 1987b, Addy, 2002, Närhi et al., 1992).The effects of DH can vary from person to person. Some individuals suffer from DH for a long time while others suffer from it for a relatively short period, even without treatment. The variation between individuals is probably due to local variables such as the formation of a smear layer over the dentine surface, occlusion of dentinal tubules by a variety of particles or other reparative measures provided by the tooth itself such as tertiary dentine formation which may seal off tubules on the pulp side (Pashley, 2013).

In addition, over time natural blockages can form inside the dentinal tubules which could explain the lower prevalence of DH in older populations. The formation of physiologic sclerotic dentine starts 2-4 years after the teeth have fully erupted. The process is characterised by the accumulation of mineral deposit within the tubules, starting at the apical part of the root and continuing coronally. It is thought this increases with age but starts when the teeth are young (Vasiliadis et al., 1983).

1.3.2 Lesion localisation and gingival recession

Where the gingival margin apical to the cementoenamel junction (CEJ) is apically displaced the cementum of the root surface will be exposed to the extent it becomes non-viable and abrades away. As a result, the radicular dentine in the root will typically be exposed leading to exposure of the dentinal tubules, this being the most common aetiology resulting in lesion localisation (Bevenius et al., 1994, West et al., 2013a).

Interestingly, both high and low oral hygiene standards can be associated with gingival recession. Patients who pursue good oral hygiene protocols have shown associated gingival recession due to a positive correlation between gingival displacement and trauma by tooth brushing. Individuals who practice obsessive brushing habits with regular brushing three or more times a day and for longer periods of time than the average population are found to be at greater risk of suffering DH due to gingival recession (Gillam et al., 1999). The highest levels of recession are intrinsically linked to the teeth that commonly receive the most attention during the brushing cycle, namely the buccal surfaces, left side, canine and premolar teeth (Gillette and Van House, 1980, Gorman, 1967). An epidemiological study of plaque distribution has shown that gingival recession was observed at similar sites to superior plaque control, sites where the least amount of plaque is found exhibit the most recession (Addy et al., 1987b).

It is believed that during any brushing cycle the toothbrush can cause a degree of scratching on oral tissue which may predispose individuals to an increased likelihood of permeant trauma and result in gingival recession (Timmerman et al., 1995, Addy and Hunter, 2003, Khocht et al., 1993, Gillam et al., 1999).

According to a 2005 Cochrane review, the use of powered toothbrushes has not been shown to cause more gingival recession when compared to a manual toothbrush if used correctly (Robinson et al., 2005). In studies aimed at simulating the effect of tooth brushing on soft tissue, most did not include the use of a toothpaste whose abrasive properties are likely to contribute to gingival recession (Imfeld and Sener, 1998, Sandholm et al., 1982). From a patient health perspective it is vital to understand that even though tooth brushing appears to cause trauma to gingival tissues leading to displacement of the gingiva, the benefits of tooth brushing to the health of the gingiva far outweigh disadvantages such as gingival recession (Addy, 2008).

Furthermore, research has shown that poor oral hygiene is a major cause of gingival recession. Individuals with poor oral hygiene tend to have higher levels of gingival recession compared to those with good oral hygiene, indicating that there are no benefits to be gained by practicing inadequate oral hygiene (Löe et al., 1992).

In addition, it has been established that periodontal disease and periodontal treatment result in gingival recession, as the remodelling of the supporting tissues around the tooth after surgery results in an apical shift of the soft tissue margin (Serino et al., 1994) . In fact, approximately half of periodontally treated patients who have had scaling and root planning experience gingival recession and root sensitivity (Von Troil et al., 2002). However, the amount of gingival recession varies based on the treatment provided. Surgical treatment tends to result in greater gingival loss due to the removal of tissue or as part of the healing process. Non-surgical treatments do not cause the same level of recession (Van der Velden and Attstrom, 1997). If we are to follow the definition of DH set out by Addy and Dowell (1983) then sensitivity arising from periodontal treatment cannot be classified as DH as an alternative pathology is present.

Anatomical factors should also be considered, individuals who are predisposed to gingival recession include those with absent or thin buccal alveolar bone (Lost, 1984). It is possible that previous orthodontic treatment may contribute to these anatomical features and that extent, severity and prevalence of recession correlates with past orthodontic treatment (Joss-Vassalli et al., 2010, Slutzkey and Levin, 2008, Tugnait and Clerehugh, 2001).

A further etiological factor may be orthodontic movement of teeth to positions outside the labial or lingual plate, which could lead to dehiscence formation. However, a systematic review that examined the incisor inclination changes during orthodontic treatment on gingival recession demonstrated that the quality of evidence was low, and more research is needed to determine the effect of orthodontic treatment on gingival recession (Joss-Vassalli et al., 2010). A piece of epidemiological research on an urban population in Brazil provided evidence that gingival recession may be associated with age and also found that the lower the socioeconomic status of the study participants the higher the prevalence of gingival recession in comparison with those in a higher socioeconomic bracket, irrespective of age (Susin et al., 2004).

The range of evidence points towards the possibility that gingival recession may be a multi factorial aetiology with no single cause responsible. Causes of gingival recession may be due to specific anatomical features such as alveolar process dehiscence, fenestration of alveolar bone or thin alveolar process. Moreover, gingival displacement may be caused by a multitude of factors such as periodontitis, gingivitis, smoking, orthodontic treatment, prosthodontic appliances like dentures that can cause trauma to the gingiva, trauma from nail biting, intra- and perioral piercing- as they may cause gingival tissue trauma, and excessive tooth brushing. It needs to be considered whether all these anatomical, pathological and physical factors act synchronously or not (Smith, 1997).

1.3.3 Loss of hard tissue exposing dentine

The coronal dentinal tissue is covered and protected by enamel and the loss of this tissue will expose dentine. The loss of enamel can occur through several different processes. Hard tissue loss may occur as a result of both carious and non-carious processes. NCCLs play an important role for dentine exposure at the cervical margin. Attrition, abrasion, erosion and abfraction are considered non-carious surface loss and all contribute to hard tissue loss. The aetiology of this process is usually multifactorial and rarely due to only one of the wear phenomena (Aubry et al., 2003, Kaidonis, 2008, Mair, 2000, Meurman and Sorvari, 2000).

Abrasion can be defined as the physical wear of hard tissue due to mechanical processes involving foreign substances or objects and is considered a major factor in the aetiology of tooth wear at the CEJ (Ganss, 2006). Abrasive processes have been established as the leading cause for dentine wear, with circumstantial evidence indicating tooth brushing with a toothpaste as the abrasive agent (Addy and Hunter, 2003, Gillette and Van House, 1980, Hirschfeld, 1939, Kitchin, 1941, Phaneuf et al., 1962). However, it is suggested that other erosive components such as dietary acid can exacerbate the abrasion process, resulting in tissue loss and tubular opening, thus leading to DH. It is believed just a small percentage of cases of DH are caused by root dentine wear only due to overzealous tooth brushing (Absi et al., 1992, Addy, 2005). Data from in-vitro studies has shown that toothpastes which meet the International Standards Organisation for abrasivity in normal use, will take many tens of years, indeed more than two thousand years to remove 1 mm of enamel (Van der Velden and Attstrom, 1997). However, toothpastes may play a role in localising sites of dentine hypersensitivity by acting synergistically with erosion to remove enamel at the cervical areas (Hunter et al., 2002).

Erosion is defined as chemical wear as a result of extrinsic or intrinsic acid or chelating agents acting on tooth surfaces not involving bacteria (Ganss, 2006). Erosion can result in permanent loss of tooth volume with a softened layer at the surface of the remaining tissue. It acts by softening the hard tissue surface followed by a continuous layer-by-layer dissolution (Lussi and Jäggi, 2008). Different biological, chemical and behavioural factors modify the effect of acidic agents on enamel. Extrinsic factors such as the consumption of acidic food and beverages (mainly), medicines and the use of some oral hygiene products can result in erosion, while gastric juices which can be caused by reflux disease, eating disorders, chronic alcoholism and pregnancy are considered intrinsic factors that can result in tooth erosion (Lussi, 2006b). The effects of extrinsic acids on hard tooth substrate are thought to be the most common and important aetiological factor for erosional tooth wear (Bartlett, 1997). Erosive drinks have been found to cause hard tissue loss over the whole of the crown of a tooth. Nevertheless, the majority of dentine is covered by thick enamel so as such, even when eroded, dentine will not be exposed (Addy et al., 2000, Scully, 2002), while on the cervical margin of a tooth, enamel is thin and exposure of dentine through loss of enamel is more likely to occur. Moreover, the acidic drinks are likely to remove the smear layer at the cervical margin, leaving the dentinal tubules patent and exposed to external stimuli (Addy and Pearce, 1994, Gillette and Van House, 1980, Newman et al., 2006).

It is important to understand that the process of NCCLs are multifactorial. Addy and Pearce (1994) together with Lussi and Jäggi (2008) showed that a few microns of enamel tissue can be lost due to the influence of an erosive challenge followed by tooth brushing with toothpaste.

Abfraction is the loss of tooth substance due to bio-mechanical loading forces. It is caused by flexure of the tooth during loading, leading to fatigue of the enamel and dentine at a location separate to the point of loading, which in turn provokes micro-fractures in the enamel and dentine (Ganss, 2006, Grippo et al., 2004). The aetiology of abfraction is not clear at this point and therefore may not exist as a separate entity; the occurrence has been attributed to abrasion, tooth flexure, and erosion or a combination of these processes. (Sakagushi and Powers, 2012).

Finally, attrition is the loss of tooth substance as a result of tooth-to-tooth contact during normal or parafunctional masticator activity and may play a role in some cases of occlusal dentine hypersensitivity, due to parafunctional habits like bruxism (Bartlett and Smith, 2000, Ekfeldt et al., 1990, Grippo et al., 2004).

Loss of dentine and enamel may be due to any single or a combination of the tooth wear processes, with the interaction between erosion and abrasion appearing to be significant factors for the majority of wear at the cervical margin and opening of dentinal tubules.

1.3.4 Lesion initiation

When dentine is exposed by lesion localisation DH can be initiated only if the tubules are exposed and patent to the oral environment from the pulp (Absi et al., 1989). There exists a direct relationship between the patency of the dentinal tubules and the smear layer. This layer on the dentine surface is formed by oral debris such as calcium, toothpaste ingredients, or through scaling and root planing procedures (Chabanski et al., 1997, Fogel and Pashley, 1993, Taani and Awartani, 2002). The depth of debris penetration into the tubules range from 1 to 5 µm (Brännström, 1982). Where dentine is exposed to an erosive challenge the mineralised peritubular dentine is removed and the tubule lumens open up (Meurman et al., 1991). A collagen matrix is left behind which can protect the underling dentine by forming a barrier which will in turn slow down further dentine erosion (Shellis et al., 2010).

The erosive challenge can take the form of both extrinsic acids found in the diet and intrinsic acids from the stomach. It has been found that most acidic drinks, citrus fruits and fruit juices together with some alcoholic beverages and many herbal teas may be responsible for the dissolution of the dentine smear layer after a few minutes of exposure (Absi et al., 1992, Phelan and Rees, 2003, Absi et al., 1989).

The removal of the dentine smear layer can lead to dentine permeability rate increase and therefore increase the possibility of DH (Prati et al., 2003). Moreover, abrasion and attrition and most of the other mechanical influences on dentine may produce or remove a smear layer (Shortall, 1981). Also, it has been stated that tooth brushing has clinically no significant effect on the dentine smear layer (Absi et al., 1992) however, the combined use of toothpastes and tooth brushing can play a role in both producing and removing a smear layer by removing the smear layer by detergent action and finally occluding dentinal tubules with the ingredients of the toothpaste (Absi et al., 1992, Addy and Mostafa, 1989, West et al., 1998, Pashley and Galloway, 1985a). These studies can explain the episodic nature of DH as brushing dentine with toothpaste can partially open and close the dentinal tubules.

1.4 Mechanism of Dentine Hypersensitivity

To establish the process by which DH occurs three main theories have been proposed; The Direct Innervation Theory, Odontoblast Receptor Theory and Hydrodynamic Theory.

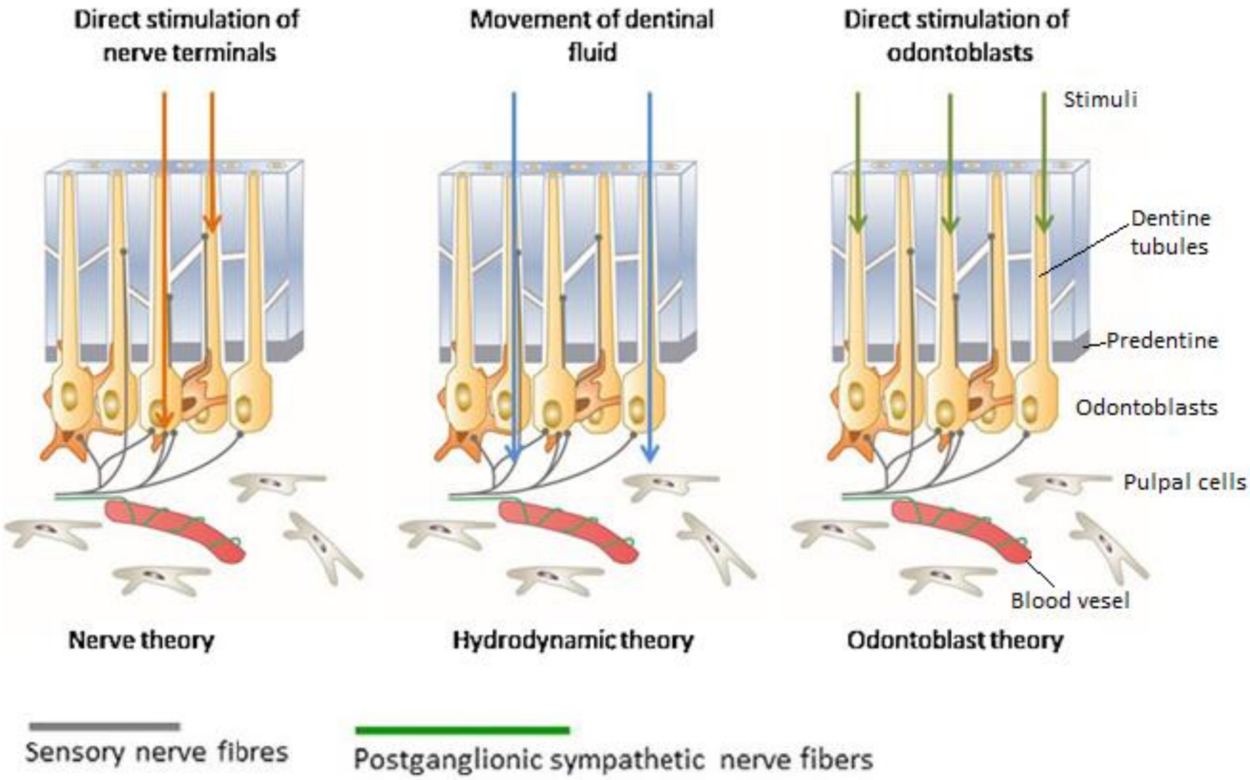


Figure 1.5 Schematic representation of the three main theories explaining dentine sensitivity (Solé-Magdalena et al., 2017)

The nerve or direct innervation theory, depicted in figure 1.5, suggests that nerve endings thread directly through the pulp into the dentine up to the EDJ whereby any mechanical stimuli would directly transmit pain through the nerves (Irvine, 1988). It was later discovered using modern electron microscopy that the nerve fibres do penetrate into the dentinal tubules but for just a short distance at the inner dentine, predominantly at the pulpal horns (Frank and Steuer, 1988). It is also possible for newly developed teeth to be sensitive, however the parietal layer of nerves responsible for transmitting pain sensation from the pulp of the tooth- the plexus of Raschkow (Scully, 2002), does not mature until tooth eruption is complete (Davari et al., 2013) which therefore puts doubt into whether this is the primary cause of DH.

The odontoblast receptor theory states that odontoblasts themselves act as the receptors of pain thereby transmitting signals to the pulpal nerves via synaptic junctions (Rapp et al., 1968). Direct microscopy has failed to confirm this as the odontoblastic processes extend only to a maximum of one half of the dentinal tubule length, stopping short of the pulpal end by 0.5-1mm (Narhi, 1985). A space can be found between the process and the tubule wall that is filled with dentinal fluid (Frank, 1968), furthermore the cellular matrix of odontoblasts are not able to stimulate neural impulses (Miglani et al., 2010). Studies have demonstrated that odontoblasts are matrix forming cells and accordingly are not considered to be excitable cells as no synapses have been established between nerve terminals and odontoblasts (Pashley, 1996).

The most prominent theory on the cause of DH is hydrodynamic theory. First proposed by Gysi in the 19th century after making a hypothesis of outward fluid flow along dentinal tubules increasing after the correct stimuli had been introduced, thereafter eliciting a response in the pulpal nerves (Gysi, 1900).

Throughout the 1960s hydrodynamic theory was solidified as the primary understanding of DH by Brännström and his co-workers in a series of studies. The theory proposes that stimulus-induced fluid flow inside dentinal tubules is responsible for nociceptor activation in the pulp (Brannstrom, 1963) and subsequent neuron activation which reports as pain (Närhi et al., 1992). The increased fluid flow induces a pressure change across the dentine which in turn activates myelinated A-β and some A-δ intradental nerves and unmyelinated C-fibres, either at pulp-dentine border or inside the dentinal tubules (Matthews et al., 2000). The sharp pain felt in a specific area when DH occurs is thought to be due mainly to A-δ nerves (Pashley, 2008).

In-vivo studies have highlighted the importance of dentinal tubules patency in facilitating fluid movement within the tubules by opening a small cavity and measuring changes in hydrostatic force following the application of a stimulus (Ahlquist et al., 1994). However, this methodology requires the recruitment of participants willing to undergo the procedure, which may not be necessary as the results may also be replicated outside of the oral cavity.

The hydraulic conductance of dentine slices or whole teeth with a section of the enamel removed can be used to quantify the amount of fluid movement through the sample in vitro, dentine with a high conductance rate has a low resistance (Berggren and Brännström, 1965). Studies performed on extracted whole teeth found that teeth which were sensitive before extraction have as many as eight times more tubules and these tubules can be two times wider than extracted non-sensitive teeth (Absi et al., 1987a) therefore providing a greater surface for sensitivity to occur. The most significant factor is likely to be the difference in tubule diameter as fluid flow is proportional to the fourth power of the radius, doubling the tubule diameter results in a 16 times increase in fluid flow (Addy, 2002).

1.5 Treatment of Dentine Hypersensitivity

It is essential to consider the individual causative factors of DH in order to prevent perpetuation of the condition. Management strategies for dentine hypersensitivity are almost entirely based on treatment needs with toothpaste formulations being developed to cover the exposed dentine and/or block the dentinal tubules (Addy, 2015). Any treatment plan should be constructed according to 1) the identification of aetiological and predisposing factors, 2) the modification, reduction or elimination of the aetiological factors, 3) the differential diagnosis provided to identify alternative or additional causes of the dental pain, 4) the correct diagnosis of the condition and 5) the provision of proven treatment based on the individual need (West et al., 2014).

Essentially treatment for DH can be classified by mode of delivery into ‘professional or in office’ or ‘over the counter or home use’. The majority of professionally applied treatment aim to occlude dentinal tubules.

1.5.1 Professionally applied treatment

Professional treatment can offer an alternative approach to reduce the symptoms of DH. Laser treatment, adhesive resin bonding, primers, sealants, varnishes, conventional glass-ionomer cements and resin reinforced glass ionomer have all been reported to show varying degrees of efficacy for the treatment of dentine hypersensitivity with some data supporting their use (Duran and Sengun, 2004, Pamir et al., 2007, Veitz-Keenan et al., 2013) and others suggesting they are ineffective (Mehta et al., 2014, Vieira et al., 2009).

A meta-analysis has shown that Er:YAG, Nd:YAG and GaA1A lasers are proving to be an effective treatment option but heterogeneity in the data suggest the need for more research (Sgolastra et al., 2013). Recently, it has been shown that Diode laser application was able to occlude 68.9% of all tubules surveyed, highlighting its possible value in the treatment of DH (Patil et al., 2017).

Considering that almost all professionally applied desensitising agents claim to reduce or eliminate pain in DH treatment process, which treatment approach is the best is still not clear and a meta-analysis of studies that have investigated the efficacy of chemical and physical occluding agents, nerve desensitising agents, laser or combination treatment concluded that all were effective when compared to placebo control (Shiau, 2012). Therefore, a variety of different methods delivering a range of different agents seem to be effective (Jackson, 2000).

A systematic review which reviewed 105 randomised controlled trials concluded that among self-administered agents, toothpastes containing stannous fluoride, arginine, CSPS and strontium were all clinically effective for the treatment of DH. However, regarding professionally applied agents, there is limited evidence to confirm which agent's efficacy was superior so it was difficult to distinguish and recommend one agent compared to another for treatment of DH (West et al., 2015).

1.5.2 Over the counter products for the treatment of DH

Alteration of fluid flow in the dentinal tubules and modification or blocking of the pulpal nerve response are the main therapeutic modalities used for the relief of pain in DH treatment (Cummins, 2009b). Toothpastes are the most common preparations used in treatment of DH and there are a considerable array of agents and compounds which have been tried and tested to characterise their efficacy for symptoms relief of DH.

1.5.2.1 Potassium

Various potassium salts have been incorporated into toothpastes to desensitise the nerves by interrupting the pulp response. Initial evidence suggested that by using toothpastes containing potassium salts for two weeks DH relief was provided, thus the toothpastes were effective (Nagata et al., 1994, Silverman et al., 1996). One hypothesis for the mechanism of action of potassium is that the body of the dentine absorbs potassium ions and eventually this can form a deposit at the pulp dentine border which blocks neural responses (Addy and West, 2013). Early studies have supported the efficacy of treating DH with potassium salts (Nagata et al., 1994, Schiff et al., 1998, Schiff et al., 1994, Silverman et al., 1996, Sowinski et al., 2000).

More recently however, some studies have demonstrated that potassium containing toothpastes do not always perform better at reducing pain when compared to control or negative control toothpastes (Boneta et al., 2013, Pradeep and Sharma, 2010, West et al., 1997). In a recent systematic review of the efficacy of potassium ions in the treatment of DH, it has been shown that over long-term evaluation there is weaker evidence for potassium ions to relieve DH when compared to arginine and CSPS, which are designed to block tubules rather than desensitise the nerves (West et al., 2015). Several studies have suggested that when other agents were compared to potassium for treatment of DH some of them were more effective. For example, in randomised clinical trials (RCTs) potassium was found to provide inferior pain reduction when directly compared to positive controls such as calcium sodium phosphosilicate (Pradeep and Sharma, 2010, Sharma et al., 2010) or Arginine (Elias Boneta et al., 2013, Kakar et al., 2012). However, the mode of action of potassium is different to calcium sodium phosphosilicate and Arginine in that it is aimed at desensitising the nerves rather than occluding the dentinal tubules.

To improve the delivery of potassium ions in toothpastes, other ions such as stannous have been added to toothpastes. It has been shown that the combination of potassium, stannous fluoride and silica in a toothpaste can be more effective at reducing pain than a toothpaste containing only potassium as an active DH desensitising ingredient (Schiff et al., 2000, Sowinski et al., 2000).

The evidence for desensitising of the pulpal nerves by toothpastes containing only potassium appears to be marginal. More often, the addition of an occluding agent leads to significant reductions in sensitivity of the participants studied; perhaps suggesting the individual effect of potassium is limited.

Certain toothpastes occlude dentine tubules to relieve the pain of DH; however, this occlusion can be superficial, dependent on the active ingredient in toothpaste and may be vulnerable to acid dissolution (Arnold et al., 2015). Below are the most widely used active agents that are administered in toothpastes to occlude patent tubules in the treatment of DH.

1.5.2.2 Stannous fluoride

It is suggested that stannous ions occlude dentinal tubules deep in the tubule which inhibits fluid movement (Miglani et al., 2010). Also, the occlusion that takes place leads to a decrease in the diameter of the dentinal tubules which will reduce the fluid flow and subsequent stimulation of the nerve (Saletta and Baker, 2005). RCTs have demonstrated the effective treatment of dentine hypersensitivity using stannous fluoride containing pastes and gel (Blong et al., 1985, Thrash et al., 1994). He et al (He et al., 2011a) investigated stannous agent's efficacy in DH pain reduction and found it to be superior when compared to the negative control sodium monofluorophosphate. Furthermore, the mode of action for stannous has also been investigated by in vitro studies which have shown that stannous fluoride leaves a deposit on the dentine surface, occluding the tubules. Importantly, this deposit was shown to be water and acid-resistant (Addy and Mostafa, 1988, Burnett, 2013, Burnett et al., 2013, Earl and Langford, 2013, Earl et al., 2010).

Stannous has shown favourable results when tested against other agents for its treatment potential in cases of DH .When a stannous fluoride containing toothpaste was compared to a positive control toothpaste containing arginine, the stannous toothpaste showed a significant superior pain reduction in both short and long-term studies (He et al., 2011b). Furthermore, where a stannous fluoride containing toothpaste has been compared to a sodium monofluorophosphate control it has been shown to provide significantly better immediate and ongoing sensitivity relief (He et al., 2011a). When stannous fluoride and arginine calcium carbonate containing toothpastes were compared in both short and long-term studies, it has been shown that stannous fluoride toothpastes had significantly superior effect reducing pain in DH treatment (He et al., 2011b, He et al., 2011c).

Stannous appears to work well when combined with other agents. It has been demonstrated that the effectiveness of toothpastes containing both stannous and potassium combined is always higher than potassium only or negative control toothpastes (Schiff et al., 2000, Sowinski et al., 2001, Sowinski et al., 2000). However, the systematic review by West et al (2015) demonstrated the quality of evidence supporting the efficacy of stannous fluoride alone or in combination with potassium in reducing the pain in DH as compared to conventional fluoride toothpastes was moderate. Furthermore, a recent systematic review by Hu et al. (2018) indicated that desensitizing toothpastes containing stannous fluoride alone or in combination with potassium are effective in relieving DH compared to negative control.

1.5.2.3 Arginine

Toothpastes containing arginine and calcium carbonate have also been shown to be effective in DH treatment by depositing calcium and phosphate particles onto dentine which occlude the tubules (Chen et al., 2015, Hall et al., 2017, Addy and West, 2013). These deposits of arginine act as a plug in open dentinal tubules and through this blocking of the tubules a reduction in the flow of dentinal fluid can be achieved and thereby relieve the pain of DH (Kakar et al., 2013, Kakar et al., 2012, Nathoo et al., 2009, Schiff et al., 2011).

Multiple clinical trials which investigated application of arginine and calcium carbonate toothpastes with 1450 ppm fluoride over a 1 week period have demonstrated the clear treatment effects offering immediate relief of pain after treatment when compared to 2%

potassium containing toothpastes (Fu et al., 2010, Li et al., 2011, Nathoo et al., 2009). Arginine has also been proven to be significantly and consistently more effective in reducing sensitivity over medium duration studies, up to a maximum of 8 weeks (Ayad et al., 2009, Docimo et al., 2009, Hegde et al., 2013, Kakar et al., 2013, Kakar et al., 2012, Que et al., 2010, Sowinski et al., 2013). Furthermore, a recent study has shown that the application of an arginine/calcium carbonate containing paste significantly reduced DH within one to two weeks of starting treatment and was continuing to reduce DH after the 11th week of treatment (Hall et al., 2017).

However, it should be noted that where studies incorporated a positive control such as strontium acetate containing toothpastes the results were mixed and showed that no toothpaste gained superiority over the other (Hughes et al., 2010, Orsini et al., 2013, West et al., 2013b). Moreover, systematic reviews have stated that arginine/calcium carbonate is an effective agent in reducing DH and may be considered as the gold standard for treatment of DH (Cummins, 2011, Cummins, 2010, Cummins, 2009a, Yang et al., 2016).

1.5.2.4 Strontium

Toothpaste formulations containing strontium chloride and strontium acetate have been available for decades. The occluding effect of strontium salts is thought to be due to its ability to absorb in to the dentine and form strontium apatite, which may occlude the dentine tubules (Gedalia et al., 1978, Ross, 1961).

More recently, Davies et al (2011) have shown with in vitro tests that strontium containing toothpaste was more robust than the arginine containing toothpaste when exposed to an acid challenge. Acid is one of the key causative factors for DH lesion initiation therefore it is very important that an agent that is able to occlude dentinal tubules can also withstand acid exposure. However, controversial reports regarding the clinical efficacy of strontium exist. Some clinical studies have demonstrated a reduction of pain perception in patients who used strontium containing pastes (Docimo et al., 2011, Mason et al., 2010). In vitro studies have shown that strontium acetate or strontium chloride can form small crystalline deposits on the dentine surface during treatment but is easily washed away by water (Addy and Mostafa, 1989, Addy and Mostafa, 1988) and by acid (Arnold et al., 2015).

Furthermore, a systematic review by Bae et al (2015) demonstrated evidence from RCTs to support the use of potassium, stannous fluoride, calcium sodium phosphosilicate and arginine containing toothpastes for the treatment of DH. However, the use of strontium containing toothpastes as a treatment for DH did not provide sufficient evidence for its use. In a systematic review by West et al (2015) the data also showed that in terms of its ability to deliver pain reduction during DH treatment, both strontium acetate and arginine produced an unclear effect and the quality of evidence regarding the effectiveness of these agents was shown to be moderate, with the need for more studies to confirm the most effective agent

Nevertheless, a study which investigated a formulation that included strontium salts with artificial silica and non-ionic detergent systems produced deposits which occluded dentinal tubules and the deposits were also resistant to dietary acids (Addy, 2015).

1.5.2.5 Calcium sodium phosphosilicate

Toothpastes containing calcium sodium phosphosilicate (CSPS) have also been shown to be effective in the treatment of DH (West et al., 2014). In-vitro studies have shown tubule occlusion by calcium, phosphate, and silica together and they appear to be water and acid resistant. CSPS precipitates onto dentine collagen as calcium phosphate and silicate, forming deposits on the dentine surface and within the dentine tubules (Cummins, 2011, Earl et al., 2011, Joshi et al., 2013, LaTorre and Greenspan, 2010, Layer, 2011).

A RCT that compared the efficacy of CSPS, potassium nitrate and stannous fluoride treatment for DH management demonstrated that although all three agents provided clinical efficacy, more significant and substantial improvement in pain relief was seen by CSPS (Sharma et al., 2010). Several RCTs have shown a statistical significant benefit of using CSPS in treatment of DH when compared to a negative control (Du et al., 2008, Litkowski and Greenspan, 2010, Pradeep and Sharma, 2010, Salian et al., 2010). A more recent study provided evidence that CSPS containing toothpaste may mineralise dentine as well as occlude dentinal tubules and that these blockages are able to withstand dietary acid challenges (Jones et al., 2015).

1.5.2.6 Oxalate

Oxalate salts have commonly been utilised for the treatment of DH. It has previously been shown that calcium oxalate crystals precipitate within dentine tubules and therefore act as desensitising agents by occluding the tubules (Greenhill and Pashley, 1981). There is some evidence to suggest that the occluding effect is enhanced when calcium chloride is applied before or after administration of potassium oxalate containing toothpaste (Suge et al., 2005). Moreover, in a study by Sharma (2013b) a mouth rinse containing potassium oxalate was demonstrated to be effective at occluding dentine tubules. Potassium oxalate reacted with naturally occurring calcium ions found in saliva within the oral cavity to form precipitates of insoluble calcium oxalate crystals. This precipitation is relatively resistant to acid and believed to block the dentinal tubules thereby decreasing hydrodynamic fluid flow within the dentine tubules, thus reducing the pain sensation (Pashley and Galloway, 1985a).

The majority of RCTs that have investigated the effect of oxalate on DH have examined the efficacy of professionally applied products with only one study evaluating a self-administered mouth rinse (West et al., 2015).

The ability of oxalates to reduce DH compared to placebo agents, professionally applied resins such as Gluma®, Seal and Protect®, Diamine silver fluoride and lasers have been investigated. The results demonstrated inconsistent efficacy where oxalate reduced pain to a similar degree when compared to controls in some cases (Erdemir et al., 2010, Gillam et al., 2004, Pamir et al., 2007, Merika et al., 2006) and less than the controls in other cases (Camilotti et al., 2012, Vora et al., 2012).

Recently there is a new emerging evidence for self-administered oxalate product that can be used in the form of a gel strip. The application of these oxalate-based adhesive strips leads to substantial and robust crystalline deposition within the lumen of dentinal tubules with the application time and number of cycles providing a strong correlation on the treatment results in vitro. The application of oxalate via a gel strip has achieved a 70% reduction in hydraulic conductance of fluid through dentine samples and this reduction was still evident after 30 days, indicating significant benefits for the treatment of DH (Hare et al., 2016b). Moreover, in vivo clinical trials have shown more than an 80% reduction in the pain of DH following use of the oxalate gel strips (Magnuson et al., 2015, Singh et al., 2015).

Finally, many toothpastes have been formulated to contain ingredients that can physically occlude dentinal tubules to a greater or lesser degree. Another approach in the development of desensitising agents was proposed by the use of various combinations of nanoparticles such as calcium phosphate (Kovtun et al., 2012), Silica (Tian et al., 2014) and nano-hydroxyapatite (Wang et al., 2014). The idea behind this approach is that nanoparticles may penetrate into dentine tubules and that these nanoparticles could act to block fluid movement within the dentine tubules. However, this enhancement has not yet been demonstrated.

1.6 Methods of Measuring Dentine Hypersensitivity

Studies investigating DH have employed a wide range of methodologies, each with their own advantages and disadvantages. An understanding of the different approaches gives a grounding to future work and may help to explain the breadth of DH prevalence rates described previously.

1.6.1 In vitro

In vitro models play an integral part in investigating the mechanism of action and potential efficacy of a desensitising agent used in DH treatment, leading to a better treatment plan (Taha, 2014). In vitro research helps the clinician to understand the physical, mechanical, and biological properties of dental materials (Krithikadatta et al., 2014).

With in vitro studies, researchers may have the freedom to immerse the samples into various solutions at varying temperatures, concentrations and acidities. Additionally, it is possible to accurately control the brushing procedures of the sample's surfaces, avoid food debris or saliva interactions taking place as well as being able to characterise the samples using various analytical techniques. In addition, the ethical considerations are also simpler due to the laboratory setting.

The greatest control of variables in studies aiming to examine DH is offered by in vitro studies as they are cost effective and useful for gaining knowledge and developing methodology prior to undertaking clinical investigations where the expense and time taken is greater. The ability to give clear reproducible evidence, standardise conditions and control experimental variance can be considered the main advantage of these types of studies (Krithikadatta et al., 2014, Yu et al., 2017). However, the main disadvantage of in vitro studies is that in regards to DH, they do not take into account the dynamics of the oral environment and daily dietary influences which are known to effect smear layer removal and widening of tubule orifices.

One of the main methods that have been used for the in vitro evaluation of aetiology and treatment of DH is imaging of the dentine tissue surface. Scanning Electron Microscopy (SEM) has been used to characterise the aetiology and treatment of DH by providing characteristic representation of the dentine surface, patent dentinal tubules and occluded tubules (Absi et al., 1992, Absi et al., 1987a). A conventional SEM scatters electrons at the sample surface and the resulting received signal provides an image of the surface. This approach is widely used, can combine high resolution, wide depth of field observation and is considered as a reproducible test method (Claydon et al., 2009).

However, the implementation of SEM needs specific sample preparation due to the fact that human tissue is non-conductive and would form a surface charge. The surface can be sputter coated but deems the technique destructive therefore, it does not allow any treatments to be carried out post imaging. Conversely, it has been demonstrated with a modern desktop SEM that it is possible to successfully record the dentine surface by mounting the sample into a holder which acts to dissipate the electrons without the need for prior coating, thereby avoiding sample degradation and allowing samples to be repositioned in-situ following examination (West et al., 2011b).

Environmental SEM is another non-destructive technique capable of imaging the sample without the need for sputter coating and it has been used to visualise the patency of dentinal tubules of the same sample before as a control and after treatment. However, the disadvantages of environmental SEM are that it is an expensive piece of equipment and therefore may not always be readily available. The samples also need to be examined soon after treatment as any delay could cause dehydration and an alteration to the section of the sample (Lussi, 2006a, Seong and West, 2015).

SEM imaging can also be used to examine dentine tubule occlusion after the application of DH treatments by using replicas taken from natural teeth at the cervical margin. These replicas are produced by applying conventional dental impression materials to the tooth under investigation. SEM will provide an inverse image of the tooth and dentine surface, facilitating visualisation of the areas of patent or occluded tubules. This method is valuable in relating tubule occlusion measured by SEM in vitro to the pain score that can be measured by the clinician in vivo (Absi et al., 1989, Seong et al., 2018).

Another technique that can provide valuable information about the surface is energy dispersive x-ray spectroscopy (EDX) which can be used in combination with SEM to identify the chemical constituents of the surface. It can also provide chemical mapping of any surface deposits which can be useful in characterising agents used to occlude dentinal tubules for the treatment of DH (Barbour and Rees, 2004, Kunam et al., 2016, Sun et al., 2014). Further techniques that can be used to characterise the surface are confocal laser scanning microscopy and atomic force microscopy, which allow the assessment of dentinal tubule occlusion in terms of surface coverage and depth of tubule penetration following treatment with different desensitising agents (Petrou et al., 2009, Rajguru et al., 2017, Zaidel et al., 2011).

Permeability studies of dentine samples have been used widely to evaluate DH treatment using different agents. These studies are based on the ability of the desensitising agent applied to the tooth surface to prevent the conductance of fluids within dentinal tubules (Taha, 2014). The Pashley cell model was developed by David Pashley (Pashley et al., 1987a) and can be used to monitor the permeability of dentine samples. In the Pashley model a dentine section is mounted into a system filled with pressurised liquid and by sealing this system, the only fluid movement is through dentinal tubules. When the hydrostatic pressure is increased to 7 kPa, the flow rate through the dentine disc is measured to give a baseline flow rate, following this an occluding agent can be applied and the conductance re-measured post treatment to show a change in flow rate. Fluid flow through dentine tubules is measured by timing the movement of an air bubble through the system. The linear movement of the bubble within the system tubing, divided by time, allows the volumetric flow rate through the sample to be calculated to provide quantitative data (Pashley et al., 1987a, Taha, 2014). The progress of the bubble through the system may be inhibited by treatments applied to samples under analysis. If the bubble stops or does not move at a consistent speed then qualitative information regarding the treatment can be established, which can be supported by other means of measuring such as SEM analysis (Hare et al., 2016b). Notably, the most important advantage of this model is that the dentine section acts as its own control.

1.6.2 In vivo

In-vivo studies are the ideal way to investigate what happens in the oral cavity, as some important parameters such as pain can only be recorded in vivo (Joshi et al., 2013, Taha, 2014). However, many issues need to be overcome when designing an in vivo study. When the samples are the participants own teeth, a preliminary in vitro pilot study should be conducted prior to the in vivo study to determine minimal levels of risk to the participant and potential harm to their teeth should always be considered (Krithikadatta et al., 2014).

The methods used and results reported from clinical trials are sometimes inconsistent and it is important to consider that the placebo effect and the Hawthorne effect may have a significant role in the results obtained and analysed by in vivo studies (Pinto et al., 2010, Rees and Addy, 2002, McCambridge et al., 2014). The comparison between different products can be difficult due to the variabilities in study methods. Additionally, standardisation methods that are normally used for in vitro studies such as preparing samples in a certain way or removing specific criteria in the study are not applicable when performing in vivo studies.

Finally, it is very important to understand the mechanism of action of the desensitising agent in reducing or eliminating DH to deliver the best treatment results. Since it is not possible to utilise many techniques like permeability measurements and SEM imaging to detect the effects of the desensitising agent, studies performed in vivo assessing DH are difficult to deliver a clear explanation of the mechanism of action (Banfield and Addy, 2004).

In order to evaluate the presence of DH, methods including the examiner-based assessment known as the Schiff cold air sensitivity scale and a subject based assessment such as the Visual Analogue Scale (VAS) are utilised. The Schiff index records the reaction to a common DH stimuli in the form of a cold air blast at the cervical margin, the score is based on the clinician's interpretation of the participants reactions which is recorded on an ordinal scale (Schiff et al., 1994). The VAS involves the participant rating the intensity of the pain response by placing a line on a linear scale 100 mm in length, the score can therefore be quantified by measuring the position of the line relative to 0 on the scale (Ide et al., 2001).

1.6.3 In situ

Models in situ can provide a bridge between the analytical techniques used in vitro and in vivo studies as they strike a balance between advantages and disadvantages of in vitro and in vivo studies. In situ study design was first described by Koulourides and Chien (Koulourides and Chien, 1992) as an experimental model for intraoral cariogenicity research and have been widely used to measure a variety of phenomena that occur in the oral environment, including hard tissue loss, remineralisation and DH (Creeth et al., 2018, Kawasaki et al., 2001, West et al., 2011a).

In situ models involve the use of tooth samples mounted into a removable appliance or other devices that will be given to a participant to place in the oral cavity which are then treated with the test product either in situ or ex situ (Banfield and Addy, 2004, West et al., 2011a, Zero, 1995). The main advantage of these study designs is that they are able to use the oral environment while maintaining the ability to use samples prepared to the specifications required by the study test techniques and closely adhering to the laboratory based experimental regulations, thereby providing superior clinical relevance when compared to in vitro studies.

One of the problems raised with in situ studies is participant cooperation. In order to overcome this problem, samples are sometimes fixed to the teeth of the participants for short periods or can even be imbedded in upper and lower denture bases or mouth guards (Amaechi et al., 2010, White, 1992, Zero et al., 2006).

For DH treatment, in situ studies have an important value in supporting the efficacy of a treatment used in a clinical study, particularly with respect to the assessment of tubule occlusion and ability of an agent to withstand dissolution and removal from the dentine tubule due to acidic dietary challenges (Banfield and Addy, 2004, Olley et al., 2012, Claydon et al., 2009).

1.7 Aims and Objectives

After reviewing the literature it is clear that products designed for relief from the symptoms of DH are primarily in the form of toothpaste to be used at home. Some products recommend a dab on approach allowing targeted application to specific teeth however the standard treatment is conducted with twice daily brushing. The active ingredients in these toothpastes may take time to build a layer sufficient to occlude the tubules and the treatment must be maintained continuously in order to be effective.

The primary objective of this investigation was to determine the efficacy of potassium oxalate treatment administered in the form of a gel strip to reduce the hydraulic conductance of dentine as a means to reduce the effects of DH. The strip allows for targeted self-application to specific teeth and the gel acts to hold the treatment product in place during application, allowing penetration of the ingredients into the patent tubules. The advantage of this application is an immediate reduction in the symptoms of DH and treatment penetration into the dentine, not just surface coverage, providing greater resistance to treatment removal. The model used included an in-situ phase to establish the resilience of the product when exposed to the oral environment and to explore the viability of using this technique for any future investigations.

The aims and objectives of this investigation were:

1. To establish which preparation procedure for dentine disc samples would provide the most consistent results when the discs were inserted into a modified Pashley cell model, named a flow cell, used to measure the hydraulic conductance of fluid flow through the dentine tubules. Specifically, the most appropriate acid etching and tooth brushing technique to be applied to the sample prior to taking flow rate measurements was investigated. Once the best preparation method was identified the secondary aim of the study was conducted.

2. To evaluate the effectiveness of a gel strip impregnated with 3.14% potassium oxalate to reduce the hydraulic conductance of fluid permeating through dentine samples using the flow cell apparatus. Furthermore, to determine the effectiveness of the gel strip in reducing fluid flow following an in situ phase where the dentine discs have been exposed to the oral environment when housed in palatal appliances worn by study participants post treatment.

3. To compare the results of the dentine discs treated with potassium oxalate to dentine discs which received no treated but were also mounted in the same oral appliance and worn by participants.

4. To verify the results found in the flow cell section of the study by using focused ion beam scanning electron microscopy (FIB-SEM) and a desktop scanning electron microscope to image the dentine surface and within the dentine tubules for evidence of occlusion by the treatment product or any other features. Energy dispersive X-ray spectroscopy (EDX) was also used to try and identify the chemical make-up of any debris deposited within the dentine tubules.

2 In vitro investigational work to determine the best etching and surface brushing methods for measuring the hydrodynamic flow through dentine using the flow cell model

2.1 Introduction

The consensus of opinion regarding the mechanism for dentine hypersensitivity favours the hydrodynamic theory which proposes that a stimulus applied to exposed dentinal tubules will result in a transient pressure change, inducing movement of fluid in the tubules and subsequent activation of mechanoreceptors with a correlated pain sensation (Brannstrom, 1963).

The Pashley model has been shown to be an accurate tool for quantifying fluid flow through dentine tubules (Pashley and Galloway, 1985a). The apparatus consists of a modified pressure cooker containing a saline solution under pressure supplied from a nitrogen canister. A dentine disc is placed between 2 rubber washers which is subsequently mounted into a custom plastic housing. The mounted sample is connected to the pressurised liquid by tubing containing a micro syringe allowing an air bubble to be inserted into the system. The hydraulic conductance of saline through the dentine sample is calculated by recording the progress of the air bubble across a micro-pipette then converting this timing data into fluid flow rate. In order to acquire fluid flow measurements from the modified Pashley cell instrument developed for this study a baseline flow rate is determined prior to treatment. Another flow rate reading can be taken following any treatment and the change in flow rate reported.

The preparation of the dentine sample is paramount to the amount of fluid flow through the sample. Sectioning of dentine samples from whole human teeth leaves a smear layer in the dentine tubules and on the surfaces of the disc which interferes with the permeability of the dentine (Pashley et al., 1981). This smear layer is composed of grinding debris formed as a result of sectioning (Scott and O'Neil, 1961), with particles varying in size from less than 1 µm to more than 15 µm (Gwinnett, 1973).

Polishing the sample can begin to remove this smear layer and give the sample a consistent thickness and surface morphology. However, polishing can lead to further penetration of debris into the dentine tubules that must be removed prior to experimentation with the sample, as the dentine sample needs to have open tubules to allow fluid flow to be measured. Etching of dentine samples has been used extensively to remove the smear layer by exposing samples to solutions such as ethylenediaminetetraacetic acid, citric acid and acetate buffer solution which leave patent tubules ready for evaluation (Shellis and Curtis, 2010, Seong et al., 2013, Santiago et al., 2006).

Following polishing and etching of the dentine sample the dentine disc surface requires a series of surface brushing stages that involve brushing the occlusal surface of the disc using an electric and then a manual toothbrush. This procedure helps to clean the surface and prevent drift in hydraulic conductance.

Preliminary data from testing the flow cell apparatus used in this study revealed some variation in baseline fluid flow rates, thought to be as a consequence of varying brushing times prior to each baseline reading. As standard the surface brushing stage involved 4 minutes manual and 4 minutes electric tooth brushing prior to the first baseline reading and 30 seconds brushing in between each subsequent baseline reading with 2 minutes of equilibration time in between each reading. Following the initial total of 8 minutes brushing the first baseline reading produced a consistently faster flow rate through the dentine than the subsequent baseline readings.

It was hypothesised that the initial extended brushing time was preventing drift in hydraulic conductance by removing more of the surface build-up of crystals that may form from the flow solution and that it may be necessary to repeat the 8 minute brushing between every set of readings. Furthermore, it was also conceived that a 4th cycle of brushing and baseline flow rates might be beneficial to provide another data point in order to provide better baseline reproducibility prior to treatment.

2.2 Aims

The aims of this investigation were:

1) To identify an acid solution best able to remove the smear layer on the dentine surface to reveal open tubules with minimal damage to the underlying dentine scaffold structure, as evidenced by providing consistently reproducible flow rates through the dentine and surfaces clean of debris as determined by SEM imaging. Any effect that different etching treatments had on the efficacy of the gel containing 3.14% potassium oxalate was also recorded to ensure that the surface had been sufficiently etched for the application of this product as indicated by a reduction in fluid flow following treatment.

2) To determine a surface brushing routine that would provide the most reproducible baseline readings prior to treatment by establishing if repeating a full 8 minutes of tooth brushing between each baseline reading would improve the consistency of flow rate readings in comparison to a 30 second brushing between baseline readings.

2.3 Materials and Methods

2.3.1 Sample preparation

Whole disinfected human wisdom crowns, from which the root and pulp had been removed, were obtained from the NHS Research Ethics Committee Northern Ireland (ORECNI) HSC REC1 approved Bristol Dental Hospital tooth tissue bank, REC REF: 11/NI/0145.

Dentine discs were prepared by sectioning the human third molars parallel to the occlusal surface of the tooth using a Micro Slice Annular Saw (Ultra Tec Manufacturing Inc, Santa Ana, USA) with direct water irrigation applied to the blade. Care was taken when sectioning the dentine disc to avoid the presence of enamel on the side closest to the occlusal plane or the pulpal horns on the side closest to the cervical margin as outlined below in Figure 2.1.

The sections were cut to 1.2 mm (+/- 0.2 mm) in thickness and it was necessary that they be >1 cm in diameter to form a seal in the flow cell apparatus. The cervical side of the disc was identified with a tape marker on the enamel surrounding the dentine to allow orientation of the sample.

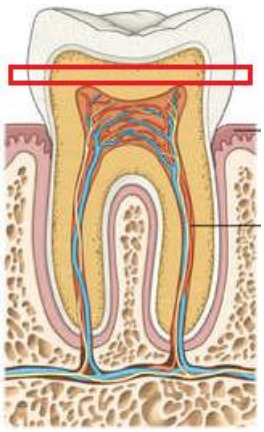


Figure 2.1 Longitudinal cross section of a human molar tooth with the location of dentine section highlighted with a red box (Farlax, 2009)

Figure 2.2 Prepared dentine disc with surrounding enamel

Both sides of the discs were polished by hand using silicon carbide paper (Kemet International Ltd, Maidstone, UK) with deionised (DI) water as a lubricant. This technique was conducted using a figure of 8 motion on P1000 grade silicon carbide paper for 30 seconds, then a P2500 grade silicon carbide paper for 2 minutes followed by repeat polishing in straight lines moving in one direction for 10 seconds. This polishing protocol was determined following previous in-house work carried out by Procter and Gamble. After polishing, the sample thickness was measured using digital callipers (Draper Tools Ltd, Eastleigh, UK) and if >1.1 mm the polishing steps were repeated. Figure 2.2 above displays the coronal side of a dentine and enamel disc section fully prepared and ready to be inserted into the flow cell.

Samples were stored in 1000 ppm chlorine solution and refrigerated at 4°C to inhibit microbial growth until they were to be used.

The final step of preparing the dentine discs was to etch the surface to remove any smear layer that had formed during the preparation stage. This is explained further in section 2.3.3 below.

2.3.2 Flow cell set up

The apparatus used in this study consisted of a custom built modified Pashley cell, named a flow cell. The development of this redesigned cell and the protocol for testing was conducted during previous in-house work carried out by Procter and Gamble.

The base of a flow cell which held the dentine sample was formed from a custom acrylic block which contained a central reservoir with channels either side to allow tubing to be inserted (Figure 2.3). The tubing brought fluid into the reservoir from one side and a second tube on the opposite side lead to a removable plug. When closed this plug ensured that the only path for release of pressurised liquid was up through the dentine sample positioned over the reservoir.

The liquid used for calculating the hydrodynamic flow rate through the dentine was Hartmann's solution (HS) (Baxter Healthcare Ltd, Norfolk, UK), a saline solution with the same tonicity as blood that mimics pulpal fluid. A wash bottle pressurised by compressed air contained and applied the pressure to the HS at 207 kPa.

An adjustable valve allowed selection of fluid flow under a pressure of 3 kPa to be used as it consummates with in-vivo pulpal pressure or 207 kPa as it represents approximate pressure reported to cause moderate pain in-vivo (Ahlquist et al., 1994). The pressurised liquid was carried by tubing leading from the regulator towards the flow cell. When the adjustable valve was switched to the off position a pressure of 3 kPa was produced via gravity by positioning the wash bottle reservoir exactly 30 cm from the bench top.

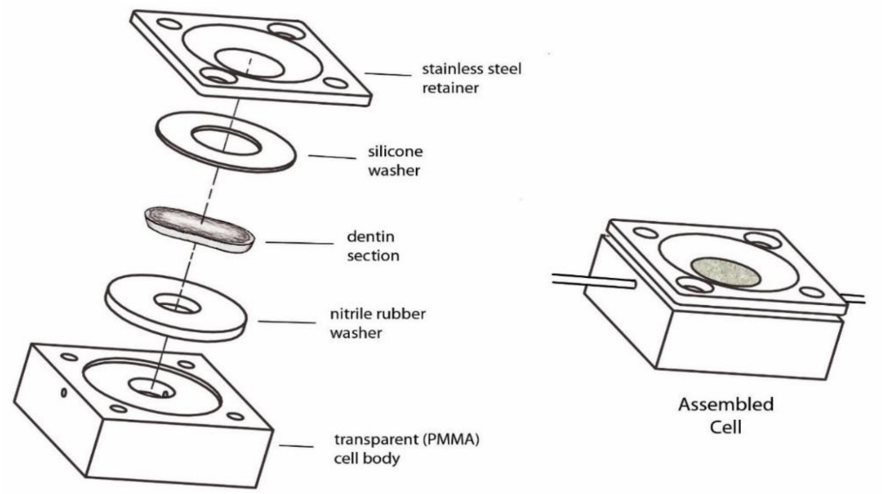


Figure 2.3 Schematic of cell layers (left) and assembled cell (right) (Hare et al., 2016a)

To assemble a flow cell the prepared dentine sample was orientated with the tape marker underneath- towards the acrylic block, so that the dentine radiated as it would in a whole tooth. The dentine disc was sandwiched between a lower nitrile and upper silicone washer then positioned centrally atop the acrylic block. The opening where the HS flowed towards the dentine disc for both the acrylic block and lower nitrile washer was 6 mm in diameter. A stainless-steel retainer was located above the silicone washer and secured with 4 corner screws that thread into the acrylic block, sealing the cell. The stainless-steel retainer contained a circular, 9.5 mm diameter opening, allowing through flow of the HS and treatment applications to be performed on the dentine. The schematic for the layers that constitute a flow cell and a fully assembled cell are depicted above in figure 2.3.

The fully assembled flow cell was mounted onto the side of a plastic container with a screw and washer mechanism allowing the cell to be fixed horizontally and to be flipped where necessary.

After preparation and mounting, each dentine disc underwent a sequence which is highlighted in Figure 2.4:

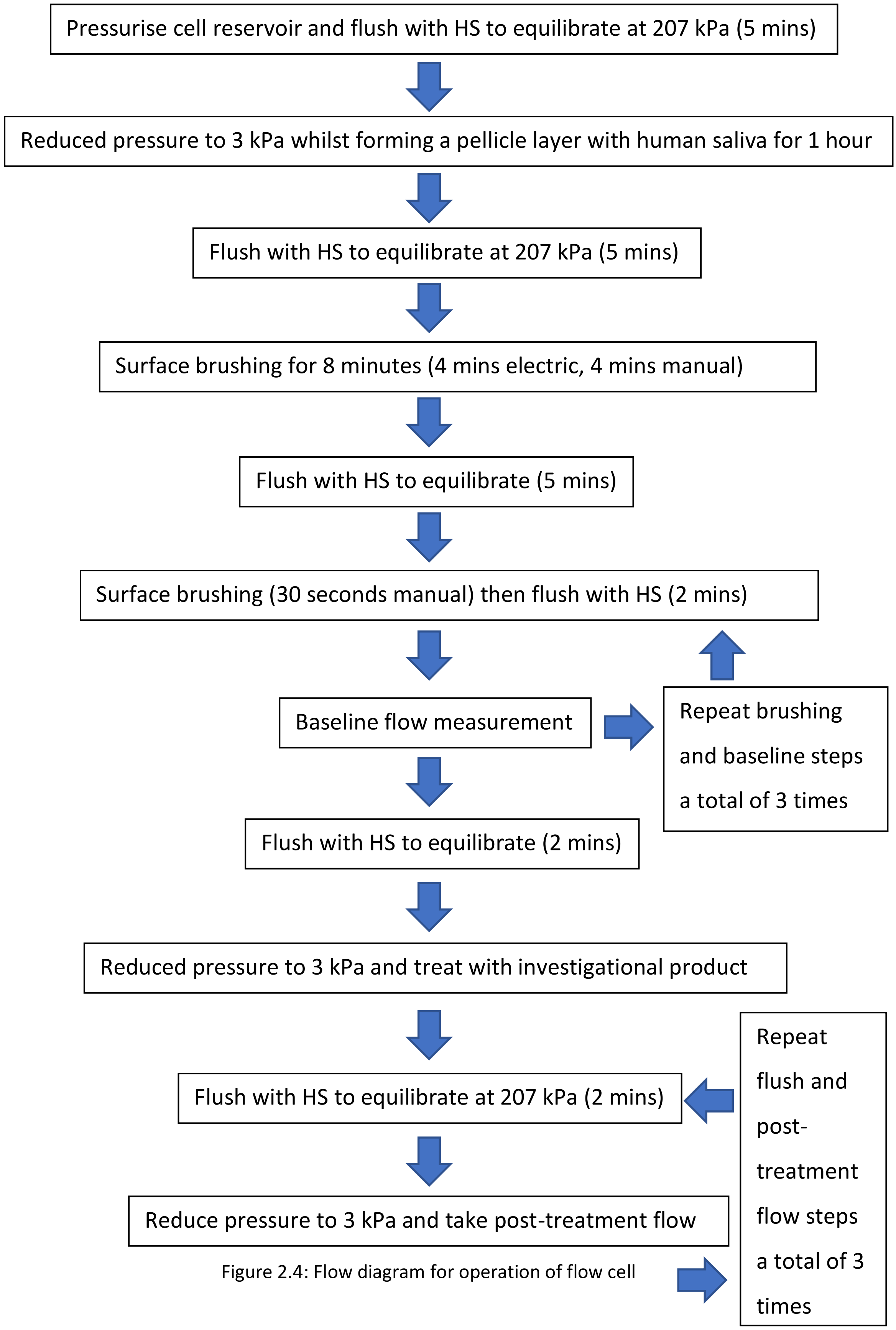


Figure 2.4: Flow diagram for operation of flow cell

With the cell connected to the system the cell reservoir began to flush with HS and bubbles formed in the cell reservoir as a result of trapped air in the tubing. The cell was flipped 180° so that the dentine faced the bottom of the plastic container and the HS flowed across the cell reservoir to remove bubbles that had formed in the tubing. The system was pressurised by turning the adjustable valve to the on position. The pressure was increased to 207 kPa with the cell in this position to avoid forcing air bubbles through the dentine, possibly damaging the dentinal tubules. As shown below in Figure 2.5, the removable plug at the end of the tubing leading from the opposite side of the cell was opened to allow the trapped air to be released before being resealed for measuring. The cell was then returned to an upright position where it was left to equilibrate with the HS running through the dentine for 5 minutes.

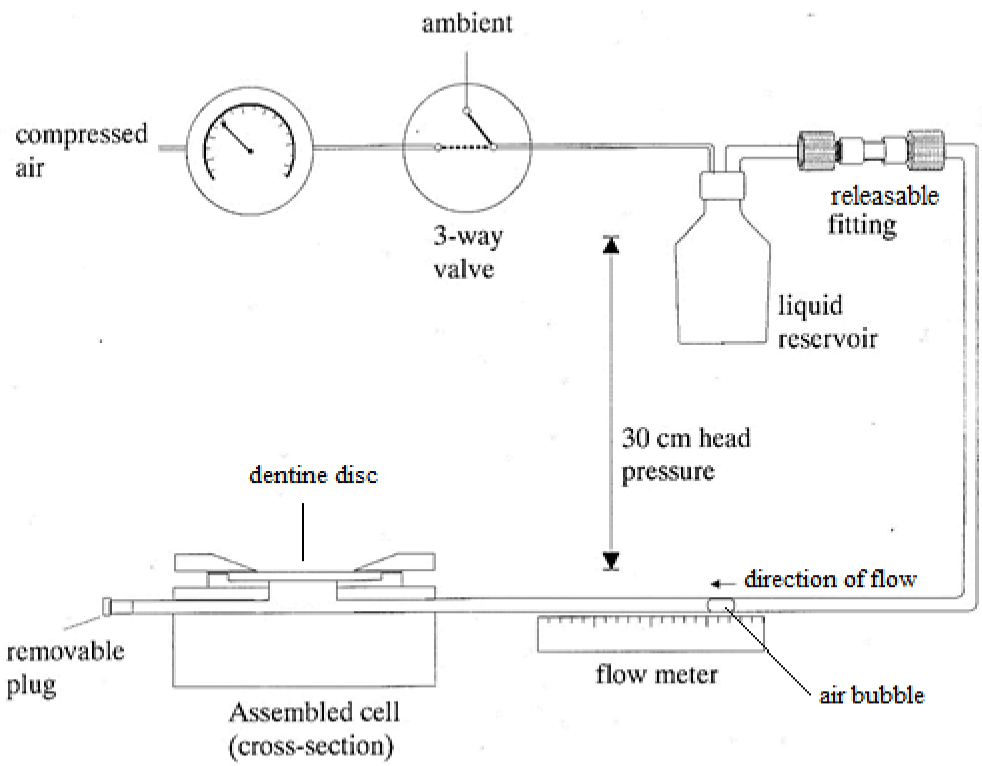


Figure 2.5 Diagram of Flow Cell apparatus (Hare et al., 2016a)

Following equilibration, the pressure was reduced to 3 kPa. The cell was tipped and any excess HS was gently blotted away from the surface with a laboratory tissue before returning the cell to a horizontal position.

A pellicle layer comprised from whole human saliva was formed on the dentine sample surface to facilitate the application of a treatment product and mimic the in vivo environment. Saliva was donated for use on the study by 6 individuals listed on a saliva bank register at Bristol Dental School. Ethical approval for the saliva bank has been provided by South Central Oxford C Research Ethics Committee REC REF: 08/H0606/87+5. Saliva was produced by chewing a wax paraffin film (Parafilm M, Bemis Inc, Neenah, US) and expectorating into a universal tube. A maximum of 15 ml was requested from donors and they were asked not to eat or drink for a minimum of 2 hours before expectorating. All donated saliva was pooled into a glass beaker and agitated. A pipette was used to transfer 0.5 ml of saliva at a time into individual eppendorf tubes before moving to a freezer for storage. The frozen saliva was removed from the freezer 1 hour prior to use and left to warm to room temperature.

The saliva was applied to the dentine by pipetting 0.5 ml of whole human saliva directly atop the dentine sample. The saliva was left to stand for 1 hour before being rinsed away with an indirect stream of HS. The cell was then flushed with HS in the same manner as above and left to equilibrate at 207 kPa for 5 minutes.

Prior to measurement, the dentine samples were prepared through a series of brushings to stabilise the sample by removing any residual deposits of HS and to prevent drift in hydraulic conductance. The dentine surface was brushed with an electric toothbrush using a back and forth motion (Oral B Vitality Plus toothbrush with Oral B Cross Action toothbrush head, Procter & Gamble Inc, Cincinnati, US) for a total of 4 minutes, pausing every 30 seconds to wet the brush with fresh HS and to rotate the direction of brushing 45°.

Brushing was then repeated with a manual toothbrush (Oral B 123 Indicator 35 medium toothbrush, Procter & Gamble Inc, Cincinnati, USA) using a back and forth brushing motion for a further 4 minutes, rotating the brush 45° every 30 seconds and rewetting the sample with HS after each rotation. In summary, each sample received a full 8 minute surface brushing cycle prior to flow rate measurement and a 30 second surface brushing in between each flow rate measurement. A consistent, gentle pressure was applied to both the electric and manual brushing technique. The pressure applied was standardised through calibration of the person performing the toothbrush by practicing brushing on a balance to maintain a weight of 120g, this was performed each day before brushing. Any bending of the toothbrush bristles during the brushing motion was kept to a minimum.

Once all the sample preparations, etching and brushing treatments had been performed a baseline flow rate reading was taken. A flow measurement was recorded by visually monitoring the progress of an air bubble adjacent to a ruler on top of a light box. The bubble was formed in the tubing by lowering the pressure to 3 kPa, loosening the releasable fitting that lead from the wash bottle reservoir followed by raising the tubing above the reservoir until a 5 cm - 10 cm air bubble formed. The releasable fitting was resealed and the 207 kPa pressure applied. The air bubble continued around the system tubing until it reached the light box and ruler. A stopwatch was used to time the air bubble as it crossed 5 distance points on the ruler. For this experiment the leading edge of the bubble was recorded every 0.508 cm over a 2.54 cm total distance. The baseline flow rate readings were repeated 4 times in the same manner. The 30 second manual brush and 2 minutes system equilibrating was repeated after each set of readings.

The linear movement of the bubble (l) within the tubing over time (t) allowed the volumetric flow rate (Q) through the sample to be calculated using the following equation:

$$Q = \frac{\pi r i^2 \, l}{t}$$

(Where r_i is the inner radius of the tube)

Comparative results are expressed as a % reduction in volumetric flow as per the equation:

$$\%\, Reduction = 100\frac{(Qp - Qb)}{Qb}$$

Where Qp = mean post-treatment flow and Qb = mean baseline flow)

2.3.3 Etching the dentine sample

To determine the etching solution that would provide the most effective removal of the smear layer whilst reducing any surface damage to the dentine sample 9 samples were prepared and equally divided into 3 groups with each group assigned to a treatment regime as follows:

- 6% citric acid solution for 4 minutes whilst under ultra-sonication (Ultrawave Ltd, Cardiff, UK), turning the sample over after 2 minutes
- 10% citric acid solution for 30 seconds, turning the sample over after 15 seconds
- 100 mM acetate buffer solution for 120 minutes, turning the sample over after 60 minutes

The citric acid solutions were prepared by dissolving the acid powder into DI water which was used as prepared and not pH-adjusted. The acetate buffer was prepared by mixing acetic acid with sodium acetate to give a 100 mmol/L solution at pH5.5. 0.98g/L of $CaCl_2.H_2O$ and 0.67g/L KH_2PO_4 were also added to the solution.

The dentine samples were separated into individual capped vials and immersed into the test etching solution for the allotted time. The samples were subsequently removed from the vial and thoroughly rinsed with DI water before measuring the baseline fluid flow rate using the flow cell apparatus as described in section 2.3.2. All 9 samples were then treated with a gel strip containing 3.14% potassium oxalate (Procter & Gamble Inc, Cincinnati, USA) as described below in section 2.3.4 before being remeasured in the flow cell apparatus allowing for comparison of pre and post treatment flow rate values.

2.3.4 Sample treatment

During treatment of the dentine sample with the potassium oxalate strip the pressure applied to the mounted dentine disc in the flow cell was reduced to 3 kPa and any excess HS was gently wiped away from the edge of the sample. A 9.5 mm diameter disc was cut from the oxalate gel strip using a gasket punch (Mayhew Steel Products Inc, Turners Falls, USA) and this was applied to the dentine disc surface. The protective plastic film was removed on 1 side to expose the gel and it was applied to the occlusal side of the dentine surface via the opening in the upper cell bracket. The application was performed with tweezers and utilised capillary action and small rotations to ensure an even spread of the gel over the dentine surface. The oxalate strip was left atop the sample for 10 minutes before being removed and the surface rinsed with DI water.

2.3.5 Sample surface brushing

The standard protocol for dentine disc surface brushing is outlined in section 2.3.2. To establish if performing a full brushing cycle between baseline flow readings would provide increased consistency in the sets of flow rate results the protocol was modified, after building a pellicle, as defined in Figure 2.6:

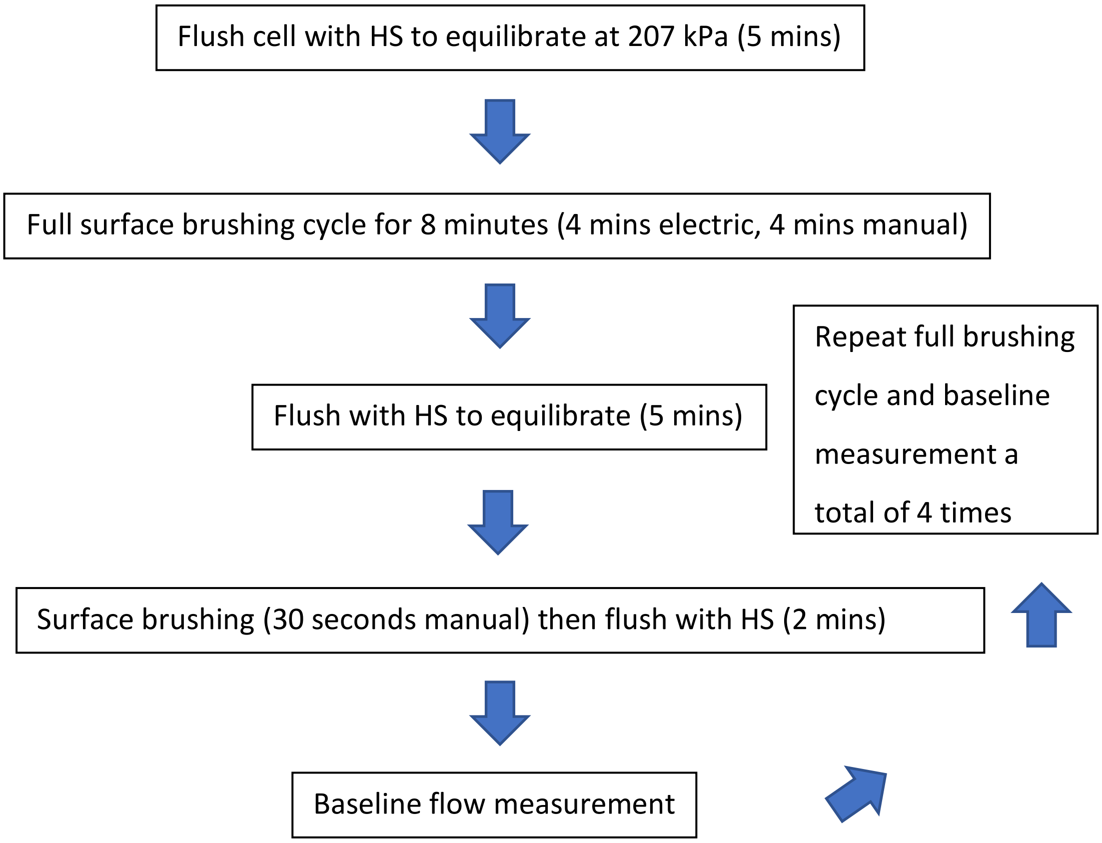


Figure 2.6: Modified sample surface brushing protocol

The revised protocol was tested on 2 dentine samples that were prepared as highlighted in section 2.3.1 and etched with 6% citric acid solution as described in section 2.3.3. Comparisons were made between the baseline flow rate that was obtained following 8 minutes of brushing as detailed above and previous data collected utilising 30 second brushing periods between each baseline reading.

2.4 Results

2.4.1 Etching results

The aim of this investigation was to choose an etching solution that would successfully remove the surface smear layer on the dentine to open the tubules and facilitate a consistently reproducible flow of fluid through the sample when mounted into the flow cell with minimum damage to the dentine structure. Treating the dentine surface with gel containing 3.14% potassium oxalate to determine how the reduction in fluid flow was affected by the different etching techniques was also investigated to confirm the dentine surface had been sufficiently etched for treatment with this product, as indicated by a reduction in fluid flow.

Samples etched with 6% citric acid for 4 minutes whilst being ultrasonicated reported a mean baseline flow rate of 69.17 µl/min. Samples etched with 10% citric acid for 30 seconds attained 50.39 µl/min and samples soaked in acetate buffer solution for 120 minutes showed a flow rate of 21.88 µl/min as highlighted below in Table 2.1.

Table 2.1: The rate of fluid flow through dentine samples etched with 6% citric acid, 10% citric acid and acetate buffer solution before treatment.

Fluid Flow Rate (µl/min)					
6% Citric Acid	Sample 1	Sample 2	Sample 3	6% Citric Acid Group Mean	6% Citric Acid Group SD
Baseline 1	38.19	86.59	117.64	69.17	28.68
Baseline 2	43.01	74.04	93.92		
Baseline 3	34.71	94	83.75		
Baseline 4	28.95	76.52	58.69		
Mean	36.22	82.79	88.5		
SD	5.92	9.24	24.42		
10% Citric Acid	Sample 1	Sample 2	Sample 3	10% Citric Acid Group Mean	10% Citric Acid Group SD
Baseline 1	49.73	20.97	133.04	50.39	35.88
Baseline 2	45.46	17.41	59.16		
Baseline 3	43.26	16.54	83.5		
Baseline 4	40.85	15.55	79.19		
Mean	44.83	17.62	88.72		
SD	3.77	2.36	31.39		
Acetate Buffer Sol	Sample 1	Sample 2	Sample 3	Acetate Buffer Group Mean	Acetate Buffer Group SD
Baseline 1	17.23	1.18	41.32	21.88	20.81
Baseline 2	22.71	1.29	44.57		
Baseline 3	21.47	1.56	43.35		
Baseline 4	23.41	1.61	42.82		
Mean	21.21	1.41	43.02		
SD	2.77	0.21	1.35		

The first flow rate taken for sample 3 in both of the citric acid treated groups was noticeably high, however this was not found in the subsequent baseline readings. Dentine discs treated with 6% citric acid achieved the highest mean flow rates across the 3 sample groups.

The flow rate readings for samples treated with acetate buffer solution were substantially lower than the flow rate of samples treated with both the citric acid solutions. The flow rates were slower for all 3 dentine discs, sample 2 in particular reached a flow rate far below what would be expected of dentine with patent tubules.

The reduction in flow rates that were measured after treating the surface with the potassium oxalate strip, in comparison to baseline flow rates, are presented below in table 2.2.

After treatment the samples etched with 6% citric acid solution showed a reduction in fluid flow by an average of 92.6 %. Samples etched with 10% citric acid solution showed a mean reduction in flow of 97.1%. Samples etched with the acetate buffer solution reduced fluid flow by 59.3% following treatment. These differences are highlighted in Figure 2.7.

Table 2.2: Baseline and post treatment flow rates of samples etched with 6 % citric acid solution, 10 % citric acid solution or acetate buffer solution.

Fluid flow rate presented as a %				
6% Citric Acid	Sample 1	Sample 2	Sample 3	Group Mean and SD
Baseline Mean	84.2	111.82	94.23	96.75
Baseline SD	13.76	12.48	26.01	13.98
Baseline %RSD	16.34	11.16	27.6	
Post Treatment Mean	5.87	6.33	9.39	7.2
Post Treatment SD	1.06	2.3	4.45	1.91
Post Treatment %RSD	18.03	36.31	47.34	
Mean % Difference Between Baseline and Post-Treatment				92.6
10% Citric Acid	Sample 1	Sample 2*	Sample 3	Group Mean and SD
Baseline Mean	98.61	101.18	125	108.26
Baseline SD	8.3	13.56	21.95	14.55
Baseline %RSD	8.42	13.4	17.56	
Post Treatment Mean	1.31	0.94	7.25	3.17
Post Treatment SD	0.42	N/A	1.4	3.54
Post Treatment %RSD	32.23	N/A	19.29	
Mean % Difference Between Baseline and Post-Treatment				97.1
Acetate Buffer Solution	Sample 1	Sample 2*	Sample 3	Group Mean and SD
Baseline Mean	93.37	109.09	96.51	99.66
Baseline SD	12.18	16.05	3.02	8.32
Baseline %RSD	13.05	14.72	3.13	
Post Treatment Mean	9.19	13.45	11.86	11.5
Post Treatment SD	1.7	N/A	1.39	2.15
Post Treatment %RSD	18.52	N/A	11.73	
Mean % Difference Between Baseline and Post-Treatment				59.3

*Sample broke after first post treatment flow rate measurement

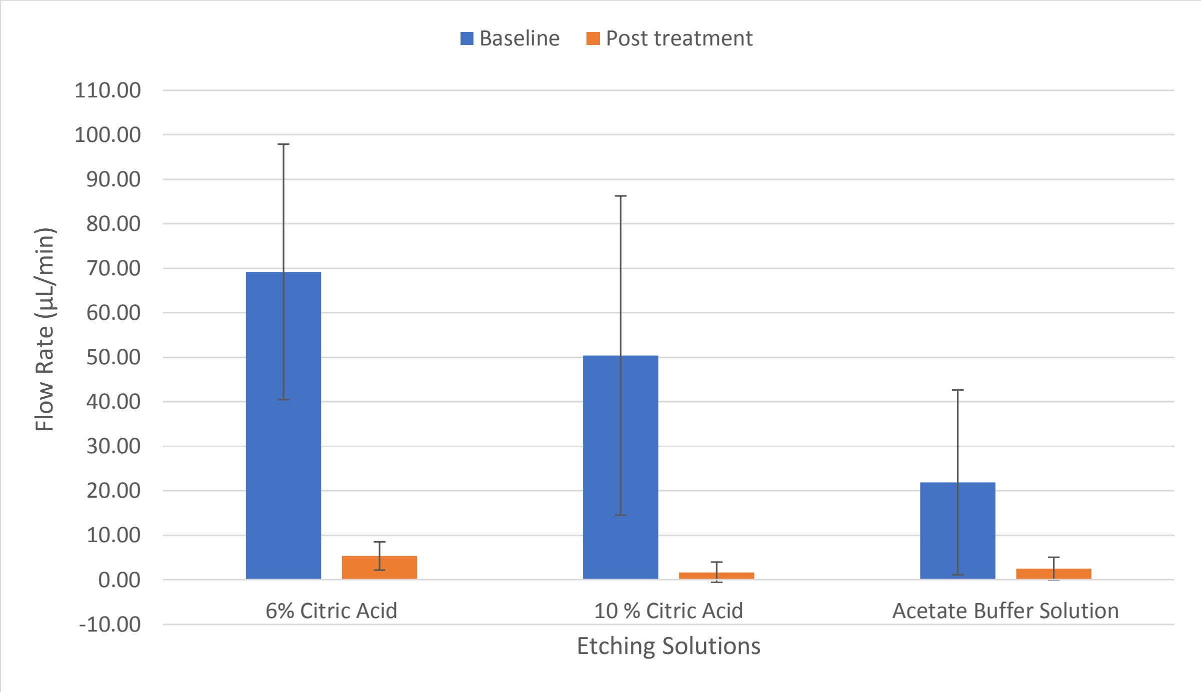


Figure 2.7 Mean flow rates for samples treated with different etching solutions pre and post treatment with gel containing 3.14% potassium oxalate

All three of the samples etched with 6% citric acid showed a substantial reduction in fluid flow following treatment with the gel strip. Sample 3 in this group displayed the most variation in the post-treatment measurements with 2 readings showing a reduction of 13% and 14% from baseline whilst the other 2 readings showed a reduction of 6% compared to baseline, however this is a relatively minor change and doesn't reflect the overall trend.

The samples treated with 10% citric acid also showed a reduction in the flow rate for all 3 samples following treatment, attaining the highest percentage decrease in average fluid flow compared to the other two methods. The first baseline value from sample 3 was considered an outlier as it was double the subsequent readings and therefore was removed from the mean calculation. Unfortunately, sample 2 broke after the first post treatment measurement was taken so only one post treatment value is available. The sample probably broke due to a crack forming in the enamel ring that enclosed the inner dentine disc.

The acetate buffer solution treated samples also reduce the fluid flow after treatment across all 3 samples however this was not to the same extent as with both the citric acid groups. Sample 2 also cracked in a similar manner to the dentine disc in the 10% citric acid group. Similarly, to the sample in the group treated with 10% citric acid, 1 set of post treatment measurements had already been recorded.

2.4.2 Surface brushing results

The surface brushing tests were performed on 2 dentine samples, 1 was brushed for 8 minutes between readings and 1 was brushed for 30 seconds between readings as outlined in the protocol above in Figure 2.6. A small sample number was used due to limitations on the number of teeth available, therefore the results are limited. Figure 2.8 below displays the flow rate for the sample that was brushed for 30 seconds between each baseline reading. After measuring the movement of the bubble over the total distance of 2.5 cm the first baseline flow rate was 12 seconds slower than the subsequent 3 sets of baseline readings which all finished within 1 second of each other. The sample subjected to 8 minutes of brushing in-between readings as displayed in Figure 2.9 showed that after measuring the movement of the bubble over the total distance of 2.5 cm the first reading had a flow rate that was 7 seconds faster than the subsequent 3 sets of measurements that all finished within 2 seconds of each other. The flow rate following 8 minutes of brushing between each measurement was substantially slower than that of the specimen that was brushed for 30 seconds in between each baseline reading.

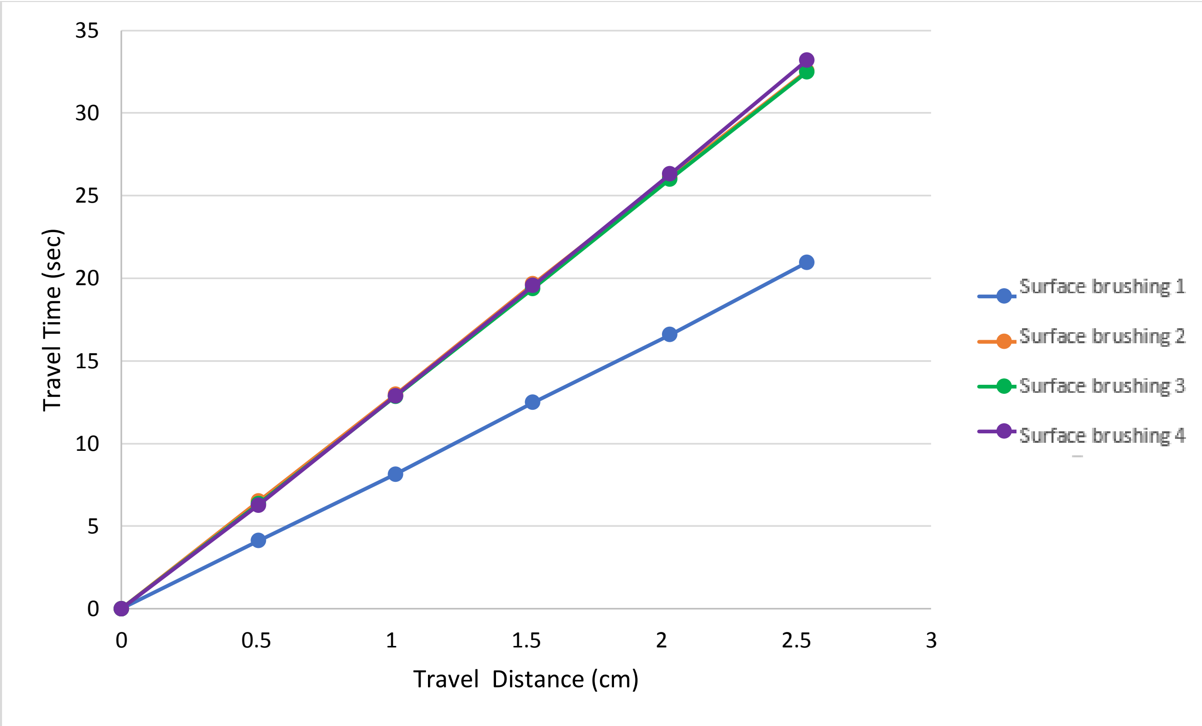


Figure 2.8 Results showing the rate of fluid flow of HS through the dentine sample using 30 second surface brushing between measurements

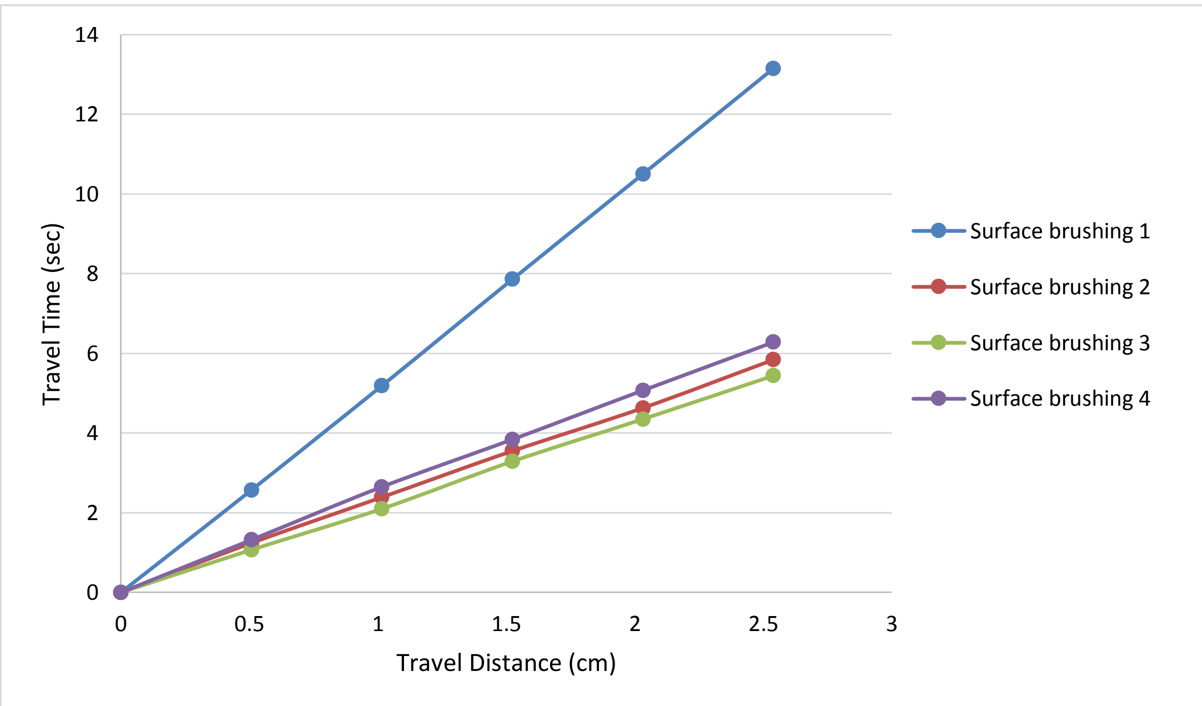


Figure 2.9 Results showing the rate of fluid flow of HS through the dentine sample using 8 minutes surface brushing between measurements

2.5 Discussion

2.5.1 Acid etching

The aim of this investigation was to establish an acid etching protocol effective at removing any smear layer present while maintaining the integrity of the dentine disk, therefore providing a suitable surface for treatment. Sectioning teeth by hand tools or rotary mechanical blades leaves behind a smear layer of cutting debris formed of partly denatured collagen and mineral (Wang and Spencer, 2002). This layer is typically 0.5-2 µm thick and can both penetrate into the tubules several micrometres and form a barrier across the dentine surface, which acts to plug the tubules and reduce dentine permeability (Pashley, 1992a).

In studies pertaining to the investigation of dentine permeability and occlusion based treatments the removal of this smear layer and opening of the tubules is vital. This is commonly achieved by the etching of the dentine samples with different solutions, time periods and agitations. In the present study 3 solutions and techniques were chosen for this purpose which have previously been successful for other in situ studies: a 6% citric acid solution for 4 minutes under ultra-sonication (Olley et al., 2012), a 10% citric acid solution for 30 seconds (Seong et al., 2013) and a 100 mM acetate buffer solution for 120 minutes (Shellis and Curtis, 2010).

The reduction in fluid flow following treatment with the gel containing 3.14% potassium oxalate for the samples treated with 6% citric acid was 92.6% compared to baseline. Meanwhile, the samples treated with 10% citric acid showed a reduction in the average flow by 97.1%. This suggests that the dentine surface had been suitably etched for treatment with the test product as the fluid flow rate reduced significantly, which is in line with previous work by Varoni (2017). Varoni reported a reduction in permeability of dentine discs treated with a potassium oxalate hydrogel of 69% following a treatment time of 10 minutes. The etching solution applied prior to treatment was ortho-phosphoric acid, which has previously been found to be more effective in removing any smear layer (Bagmar *et al.*, 2013). However, as the citric acid etching in this study showed a good baseline flow rate and the present study formed the basis for a future in situ investigation, citric acid was used to remove the smear layer as it would better mimic an everyday dietary challenge.

In the study by Hare et al (2016b) a 6% citric acid solution was used to etch the dentine discs (n=20). The discs were placed into an ultra-sonicator during the etching process and following treatment a significant reduction in flow rate of 70% from baseline levels was recorded. In the present study, a greater reduction using the same etching technique was achieved. Possibly this was due to morphology of the specific samples allowing for greater penetration of the potassium treatment into the dentine tubules. It should be noted that the sample numbers were smaller (n=3) therefore not accounting for as much variation in individual sample morphology where a mean flow rate was calculated.

In the present study, the 2 groups that had been treated with the citric acid solutions achieved such similar results that an attempt was made to further differentiate them by looking at 1 sample from each group under SEM for signs of surface demineralisation as one of the aims was to minimise damage to the dentine structure. The enamel surrounding the dentine disc became brittle when placed under vacuum and therefore the samples were taken out of the SEM before any images could be recorded to avoid damaging the instrument. Whilst the samples were in the SEM, it was noted that the sample etched with 10% citric acid displayed a rougher topography, possibly indicative of a greater demineralised surface compared to the sample that had been etched in 6% citric acid solution, however this result was not conclusive. In another study where SEM was used to investigate the effect of using 10% citric acid for etching the dentine surface, evidence was found of deep grooves and erosive effects on dentine tubules, together with significant removal of calcium ions (Reis *et al.*, 2008).

The acetate buffer solution etched samples were placed into the solution for 120 minutes (turning the sample over after 60 minutes). In other studies this has been found to successfully remove any smear layer and open the dentine tubules with minimal damage to the underlying dentine (Shellis and Curtis, 2010). In this investigation the average fluid flow was recorded as 9.30 µl/min which is markedly less than both other groups tested. The samples in this group also reported a reduction in fluid flow after treatment with the potassium oxalate containing strip of 59.3% from the baseline average, however this was far less than both citric acid groups. If acetate buffer solution were to be used in this study the etching tests would need to be run on a greater number of samples to give a better idea of how the solution etches the samples. Due to limited tissue resources it was not possible to assign more teeth to this investigation therefore this solution was not used for any further part of this project.

The variation in baseline flow rates could to some degree be attributed to the physical makeup of the specific samples assigned to that group as every sample was sectioned from a separate tooth and therefore the number of tubules accessible for fluid to flow through may vary. It is therefore difficult to completely affirm that one of the citric acid etching solutions was more effective than the other at removing the smear layer without carrying out further work.

It was unfortunate that 2 of the dentine discs under investigation formed cracks in the enamel ring surrounding the central dentine disc before all measurements were completed. As this occurred after the first post-treatment reading had been taken for both samples it was decided not to devote further tissue to replacing them. When sealing the dentine discs into the flow cell the upper stainless steel retainer is secured in place as pictured in Figure 2.3. Great care must be taken not to overtighten the 4 corner screws holding the retainer in place as the sample can be damaged as we believe happened in this case. A valuable lesson was learned in losing these 2 samples which was applied to all further work.

A 6% citric acid solution is commonly used in the literature relating to the etching of dentine and is noted for the ability to increase permeability across dentine (Pashley *et al.*, 1981). This investigation supported these findings by using a 6% solution to achieve the highest mean flow rate. It is possible that the 10% citric acid solution was demineralising the dentine surface to a greater degree without benefitting the flow rate. The 6% citric acid solution was chosen for future work in this study. Samples etched in 6% citric acid achieved consistent flow rates with substantial reduction after treatment whilst more likely maintaining the structural integrity of the dentine surface.

2.5.2 Sample surface brushing

This investigation aimed to establish the most suitable method to achieve reproducible and consistent flow rates through the dentine disc samples. Preceding the measurement of baseline flow rates, the dentine surface underwent a series of brushings to stabilise the sample by removing any residual deposits of HS and prevent drift in hydraulic conductance. Previous work carried out in our laboratory showed that baseline flow rates were producing a consistently faster flow rate through the dentine than the subsequent baseline readings, perhaps due to the extended amount of sample brushing time (8 minutes) prior to the first reading in comparison to the brushing in-between flow rate measurement of 30 seconds. Consequently 8 minutes of brushing between every set of flow measurements was tested to see if the variation between the flow rates could be reduced.

Both samples analysed in the revised surface brushing protocol showed that the first reading taken produced a variable flow rate in comparison to the subsequent readings that remained close in their timings. Performing the full cycle of 8 minutes brushing between readings for a total of 32 minutes had a detrimental effect on both the silicone washer and the dentine disc itself. The upper silicone washer used to seal the dentine disc into the flow cell started to deteriorate which would eventually lead to leakage. The sample became translucent in colour, not seen in any previous work, suggesting an over saturation of the sample with the HS. As the first flow rate remained variable it was decided that there was no benefit in performing the full 8 minutes brushing between flow rate measurements. The sample brushed for 30 seconds between readings did not display any signs of this degradation, even after including a 4th set of flow rate measurements.

The inclusion of a 4th cycle of flow rate measurements with 30 second brushing in-between provided an extra data point to improve the mean flow rate measurement data without any negative effects on the dentine sample. Also, as the flow rate measurements were taken with a stopwatch by eye, adding an extra data point served to remove some of the minor variation in timing measurements due to human error. In a study conducted by Hare et al (2016b) a similar surface brushing cycle was applied to dentine discs where if the 1st flow measurement varied by more than 5% from subsequent flow measurements, the baseline flow was calculated as an average of the three flow measurement cycles, possibly suggesting a similar issue to the present study where the 1st flow rate result varied.

The further work in this study continued to employ a 30 second surface brushing cycle between readings as outlined in Figure 2.4 but also included an additional measurement for both pre and post treatment flow rates.

3 Determining the effectiveness of an oxalate strip in reducing hydrodynamic flow rate through dentine in situ as measured by a flow cell model and surface imaging.

3.1 Introduction

The most commonly accepted theory for the mechanism of dentine hypersensitivity is hydrodynamic theory. The evidence that an outward flow of fluid through dentinal tubules existed was originally proposed by Gysi (1900). This work was much later developed by Brännström and his colleagues through a series of studies inducing fluid flow through dentine tubules in whole extracted teeth by applying various stimuli to exposed dentine sections and measuring transient movement in the fluid contained within the tubules (Brannstrom, 1963).

Building upon these foundations Pashley and his team developed a model to measure the hydraulic conductance of dentine tubules by quantifying the fluid permeating through dentine discs with the stimuli element replaced by pressurised liquid (Pashley et al., 1987b, Pashley and Galloway, 1985a). Data was collected by measuring the time taken for a bubble introduced into the system tubing to pass through a micropipette across a fixed distance and the fluid filtration rate calculated. The bubble velocity and time were the variable with the liquid pressure, micropipette diameter and length as the constant (Pereira et al., 2005). Toothpaste treatments were applied to the dentine discs before re-measuring the fluid flow rate and expressing the hydraulic conductance as a percentage of the pre-treatment values. It is this model that the present study replicates with some modifications.

The implementation of oral appliances for in situ studies provides a mounting point for samples to be housed in the oral environment. Several investigations have tested the occluding properties of toothpaste applied to dentine samples which have been inserted into intra-oral appliances, most often situated on the palate or in the buccal aspect of the lower teeth (Seong et al., 2013, Olley et al., 2012, Han et al., 2014). Intra oral appliances have also been used to test the remineralisation potential of different agents applied to dentine (Gernhardt et al., 2007, Bizhang et al., 2015, Jones et al., 2015) in situ. The present study proposed to adapt a palatal oral appliance based on a similar design to the one used by Hooper et al (2014) but with the additional requirement of needing to house larger dentine discs required for analysis employing the Pashley model as described above in Chapter 2.

The treatment to be investigated in this study was a strip coated in a gel containing 3.14% potassium oxalate. Several in vitro studies have been successful in confirming the occluding potential of this agent as indicated by a reduction in permeability through a dentine sample using a Pashley model (Hare et al., 2016b, Santiago et al., 2006, Varoni et al., 2017). However, this study sought to introduce an in-situ element to test the resilience of the treatment when exposed to the oral environment over an extended duration.

The implementation of SEM imaging for studies investigating dentine tubule occlusion can provide both an overview of the dentine surface morphology and quantifiable data where an image scoring system can be employed. Numerous in situ studies have made use of SEM micrographs from a top down perspective to allow comparisons of the degree of dentine tubule occlusion by agents administered through toothpaste and mouthwash, these can be analysed through counting of occluded tubules (Kulal et al., 2016, Rikame et al., 2018) or by scoring the degree of tubule occlusion using examiners who are blinded to the study treatment (Olley et al., 2012, Seong et al., 2013).

The ability to prepare cross sections through both hard and soft material such as dentine has been improved over the last decade by the introduction of focused ion beam scanning electron microscopy (FIB-SEM). The FIB mills away sections of dentine or other material to form a trench in the sample surface allowing a longitudinal view of dentine tubules and the contents therein (Earl et al., 2010). This technique may be used in conjunction with energy dispersive X-ray spectroscopy (EDX) to build a chemical profile of exposed surfaces and an indication of the contents of the dentinal tubules (Mathew et al., 2017).

Another procedure for achieving a longitudinal view of dentine tubules is to fracture and vertically mount a sample prior to SEM imaging. This can be achieved through the use of a chisel struck whilst in contact with the dentine (Eliades et al., 2013) or through snapping the sample with the use of forceps (Hare et al., 2016b).

Visualising the tubules longitudinally is advantageous in allowing us to gain further information about the degree of occlusion that may be taking place within the tubules. Some occluding particles such as stannous fluoride and potassium oxalate have previously been shown in vitro to form or penetrate within the tubules rather than just being visible on the dentine surface (Hare et al., 2016a, Earl and Langford, 2013).

3.2 Aims

The aims of this investigation were:

1) To determine the effectiveness of a gel strip impregnated with 3.14% potassium oxalate to occlude dentinal tubules relative to a control sample which had no treatment. A modified Pashley cell (named a flow cell) was used to determine the hydraulic conductance of dentine samples at baseline and post treatment for those that were treated.

2) In addition, this study sought to investigate the durability of the potassium oxalate treatment following an in situ phase where dentine samples were housed in the oral environment for 14 days. Any reduction in fluid flow through the treated and non-treated dentine after 14 days wear was compared.

3) These aims were supplemented by the use of SEM imaging to corroborate the flow cell data. The objective was to examine features within the untreated samples and the treated samples to establish the tubule occlusion efficacy of potassium oxalate strips when applied to the dentine discs. FIB-SEM allowed for longitudinal images of the dentine tubules to be taken and EDX provided chemical mapping of sites within the dentine to identify the make-up of any precipitates. Following the use of FIB-SEM and EDX, the aim was further pursued with the use of a fracturing technique applied to the dentine discs to allow for longitudinal observation of the dentine under desktop SEM. The objective was to capture representative images of the dentine tubules to allow for scoring of occlusion by independent examiners. An assessment of the dentine surface and any debris deposited following the in-situ phase of the study was also conducted at this stage.

3.3 Materials and Methods

3.3.1 Overview of study design

The study was conducted in the Clinical Trials Unit at the Bristol Dental Hospital with ethical approval awarded by REC: South West - Exeter Research Ethics Committee, under the REC REF: 16 SW 0050.

This was a single centre, negative control, split mouth design study. The participants were blinded as to which sample was treated or untreated in the appliance.

Female and male participants at least 18 years of age who were in good health were recruited from Bristol Dental School and Hospital, Bristol University, UBHT Trust and the local area.

3.3.2 Identity of investigational products

Test Product: Crest ® Sensi Stop Strip (1 cm x 3 cm). Gel strip contains: Aqua, Glycerin, Cellulose Gum, 3.14% potassium oxalate, Carbomer, Sodium Hydroxide, Sodium Benzoate, and Potassium Sorbate

Participant at-home product: Crest Decay Protection® (1450ppm F) Toothpaste

Oral-B® Indicator Toothbrush

(All products supplied by Procter & Gamble Inc, Cincinnati, USA)

3.3.3 Sample preparation

The dentine samples were prepared following the same method as previously described in section 2.3.1.

Following sectioning the smear layer was removed from the discs by placing into an ultra-sonicator (Ultrawave Ltd, Cardiff, UK) for 4 minutes in a 6% citric acid solution, used as prepared and not pH-adjusted, before being thoroughly rinsed with DI water.

The sample surface brushing procedure as outlined in section 2.3.2 and Figure 2.4 was followed to prepare the dentine for treatment.

3.3.4 Sample Storage

Dentine samples were stored in 1000 ppm chlorine solution and refrigerated to inhibit microbial growth. The chlorine solution concentration was reduced to 125 ppm 7 days before use. In the 48 hours before use the discs were rinsed and stored in DI water which was changed every 12 hours to flush the sample of any residual chlorine.

3.3.5 Design of appliance

The oral appliance used to house the dentine discs under investigation was designed in conjunction with the Orthodontic production laboratory at Bristol Dental School and Hospital, UK. Several prototypes were manufactured with different designs for containing and protecting the dentine discs when in-situ. The design pictured below in Figure 3.1 was chosen as it was felt the 4 parallel bars would contain the sample and allow interaction with consumed food and drink whilst protecting the dentine from large food debris. The dentine discs were held in place using a non-toxic sticky wax (Kemdent, Swindon, UK).

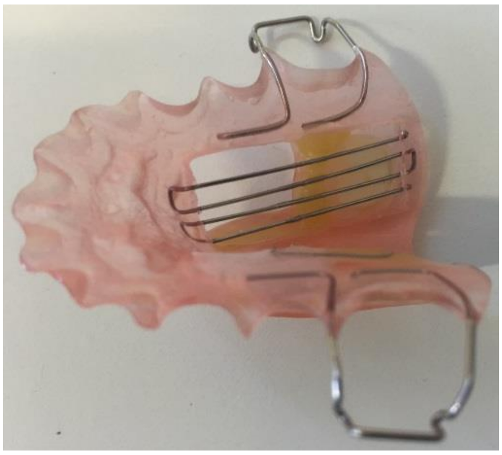


Figure 3.1 Side view of oral appliance with mounted dentine disc (right)

3.3.6 Participant Screening

Prior to receiving study specific procedures, a total of 20 individuals were asked to read a patient information sheet and sign duplicate copies of an informed consent form before being screened for suitability for the study.

Demographic information and inclusion and exclusion criteria were assessed. Participants were shown a product and ingredient list prior to any product use. Participants were given one signed copy of the informed consent and the other signed copy was kept as site source documentation. Personal medical history and concomitant medication information was reviewed and retained as site source documentation.

The participants were selected by the study clinician based on their capacity to accommodate a palatal appliance that could be mounted securely to their teeth and would be large enough to house 2 dentine discs. From the 20 people screened all 20 were invited to participate in the study.

An impression was taken of their upper teeth and palate and a palatal appliance was constructed to house the dentine samples. Each participant was supplied with a kit box containing the at home toothpaste and toothbrush with instructions for use, these instructions can be found in appendix I and II. The participants were instructed to brush their teeth twice a day, morning and night using their usual routine for the duration of the study period. Participants had used the same toothpaste for 4 weeks prior to screening during a supplementary part of this study.

3.3.7 Inclusion criteria

In order to be included in the study, each subject had to:

1. Be at least 18 years of age
2. Sign an informed consent form and be given a copy
3. Be in good general health as determined by the Investigator
4. Agree to delay any elective dentistry, including dental prophylaxis, and to report any dentistry received during the course of the study
5. Agree not to participate in any other oral care study for the duration of the study
6. Agree to refrain from the use of any non-study oral hygiene products
7. Agree to return for scheduled visits and follow all study procedures

3.3.8 Exclusion criteria

Subjects were excluded from study participation due to:

1. Periodontal disease
2. Any medical condition requiring pre-medication prior to dental procedures
3. Any diseases or conditions that might interfere with the subject safely completing the study
4. Inability to undergo study procedures
5. Fixed orthodontic appliances
6. A history of kidney stones
7. Self-reported pregnancy or nursing
8. Have a history of allergies or hypersensitivity to ingredients in commercial dental products, such as potassium oxalate, potassium sorbate etc

The participants were requested to wear the inter-oral appliances housing the dentine samples for a period of 14 days.

3.3.9 Palatal appliance fitting with baseline and post-treatment dentinal fluid flow rate measurement

The participants returned to the study site following the construction of their oral appliance. The visits were staggered over 14 days with 2 or 3 participants attending the baseline visit daily to allow time to analyse the dentine samples.

Participants were asked to try in their appliance for comfort and fit. The study clinician made small adjustments to the metal clasps holding the appliance in place to ensure a secure but comfortable fit. Any substantial changes such as removing acrylic from the body of the appliance were made by technicians in the orthodontic laboratory.

The study clinician performed oral soft tissue examinations on the participant together with recording current and concomitant medications in the study source documents.

The participants were requested to provide 5 ml of unstimulated saliva by expectorating into a universal container. The saliva was used within 30 minutes of donation as part of the sample surface brushing protocol to build a pellicle layer on the 2 dentine discs assigned to that individuals palatal appliance, therefore the saliva was not pooled together as in previous work.

In the laboratory, baseline flow rate measurements were taken using the flow cell model as described in section 2.3.2 followed by treatment of the samples as described below. Following treatment, the post-treatment flow rate measurements were taken. The dentine discs were then mounted into the oral appliance with the surface of the dentine sectioned closest to the occlusal plane facing outward towards the oral cavity and secured in place with sticky wax, making sure not to cover any of the sample surface.

The loaded appliance was submerged into a bactericidal Hydrex Pink 0.5% Chlorhexidine solution (Ecolab Ltd, Minnesota, US) for 20 minutes before being rinsed with DI water. Following this the appliance was submerged into Corsodyl Daily Mouthwash 0.06% Chlorhexidine solution (GlaxoSmithKline plc, Brentford, UK) for 2 minutes to clean the appliance and give it a fresh taste. Finally, the appliance was rinsed thoroughly with DI water before being returned to the participant. The appliance placed in situ with the dentine discs loaded can be seen below in Figure 3.2.

Participants were provided with a kit box containing the at home toothpaste, 2 toothbrushes and instructions for care of the appliance. 1 toothbrush was allocated for the participants to clean their own teeth and 1 for cleaning the appliance. It was requested that participants avoid consuming sticky and very chewy food when wearing the appliance to protect the dentine and appliance from damage but there were no other dietary restrictions. The participants were also informed of a daily appliance cleaning regime performed by study site staff at a location convenient to the participant. The daily cleaning regime consisted of cleaning the appliance using a manual toothbrush and water to remove any food deposits whilst making sure not to directly brush the dentine disc or remove the wax holding the samples in position. The appliance was submerged into Corsodyl Daily Mouthwash for 2 minutes and rinsed with DI water before being returned to the participant. Several participants attended the study site during the in situ phase to have minor adjustments made to their appliance for comfort and fit.

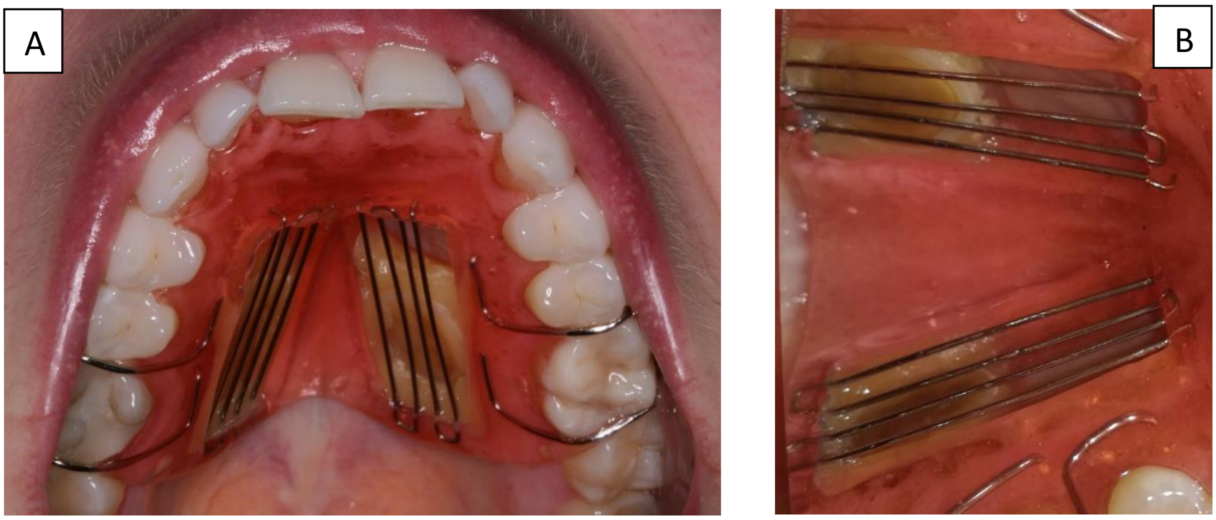


Figure 3.2: A) Palatal appliance in participants mouth with dentine discs in situ and rear brackets holding appliance in place B) Close up of dentine discs in the appliance with surrounding wax to hold discs in place.

3.3.10 Treatment

Each oral appliance was allocated 2 dentine discs. Due to some variation in appliance shapes and disc sizes the disc was checked for fit in the appliance prior to treatment and testing. For baseline measurement, the dentine sample was mounted into the flow cell as described in section 2.3.2. The surface brushing, treatment and measurement procedure was followed as outlined in Figure 2.4 however the 4th surface brushing and measurement cycle was included as described in Figure 2.6.

The saliva used to build a pellicle layer on the dentine was not pooled in this experiment but was collected from each individual participant to be applied to the specific samples that would be mounted into their oral appliance. 0.5 mL of collected saliva was pipetted onto the dentine disc.

The surface brushing and measurement procedure was completed for both samples allocated to each appliance. Following the 4th baseline flow rate measurement one of the dentine samples was treated with a gel strip containing 3.14% potassium oxalate in the manner described in section 2.3.4.

Following treatment, the flow rate measurements were retaken using the procedure detailed in Figure 2.4 before both samples were mounted into the appliance and given to the participant to wear.

3.3.11 Final visit

Participants returned to the study site after 14 days of wearing the appliance. The appliance was returned to the site along with the supplied kit box. Current and concomitant medications were recorded and a final oral soft tissue examination performed by the study clinician. This concluded the participants contribution to the study.

The dentine discs were removed from the appliance and refrigerated in universal tubes containing moist cotton wool rolls soaked in DI water to prevent the dentine from drying out prior to taking post-appliance flow rate measurements.

3.3.12 SEM imaging and EDX analysis

The dentine discs were prepared for imaging after all the flow rate readings had been taken following the oral appliance housing phase of the study presented above. 40 samples were allocated to the in situ study and of those 16 oxalate treated dentine discs and 17 untreated dentine discs remained intact and were available for analysis.

Initially 8 representative samples were selected for examination using FIB-SEM to establish the suitability of this technique for examining dentine tubule contents. 6 of the samples imaged had been treated with gel containing 3.14% potassium oxalate and 2 had received no treatment. Limited evidence exists for the use of this technique on dentine, previously treated samples were prioritised to aid the proof of concept. The samples were chosen as their flow rates represented average readings for the treated and non-treated groups.

The dentine discs were stored in an airtight container together with cotton wool rolls soaked in DI water to provide a moist atmosphere and avoid the samples drying out or becoming brittle both before and after analysis. The samples were mounted horizontally onto an aluminium stub with the occlusal side of the dentine facing upward as this was the side which had received treatment and was facing outward when mounted in the oral appliance. The samples were sputter coated (Emitech SC7620, Quorum Technologies, Laughton, UK) using gold-palladium to ensure the surface was conductive.

The samples were left to air dry at room temperature for 5 minutes prior to loading into the FIB-SEM (FEI Helios Nanolab 600, FEI Company, Hillsboro, US). The ion source used for the milling was gallium liquid-metal and the electron beam voltage for the SEM was 15 Kev with a current of 2.7 nA. For each sample under investigation a scan of the surface was made to locate a suitable position for imaging. A site was chosen that was most representative of the surface features noted by the examiner and that could cover a cross section of several dentine tubule openings. The FIB-SEM first milled a trench approximately 40 µm wide and 20 µm deep at the chosen location to provide a longitudinal view of the tubules. A platinum coating was applied to give the newly exposed surface the conductivity necessary for imaging. Images were recorded of the inside of the tubules and if required the trench size increased to further expose the tubules. If any occlusion was identified inside a tubule, EDX analysis of the formation was conducted at several sites on and around the area using a spot size of 1 µm laterally and 1 µm in penetration depth. A spectrum was compiled at each site depicting X-ray counts against energy (in keV), with energy peaks corresponding to the elements present at that site expressed as a percentage of the volume total.

In addition 33 samples were also prepared so that they could be viewed under a desktop SEM (Phenom G2 Pro desktop SEM, Phenom World BV, Eindhoven, The Netherlands). Each dentine disc was fractured to allow longitudinal imaging of the tubules. In order to fracture the dentine, the disc was attached horizontally to the bench top using double sided tape and a flat head screwdriver placed centrally at a 45° angle on the outer rim of the enamel surrounding the dentine. With a short sharp hit to the screw driver handle with a hammer the sample fractured across the centre of the dentine surface producing 2 halves of approximately equal size and half-moon in shape. The fractured sample was then mounted vertically onto an aluminium stub using a vinyl putty to hold the sample in place (GC America, Alsip, USA). The fractured surface faced upward, allowing a longitudinal view of the dentine tubules. The two halves were then re-sputter coated in gold palladium before loading into the SEM.

The entire length of the treated edge of the fractured dentine surface was viewed under SEM and representative images were taken of the tubules up to 40 µm in depth from the surface. Any occlusion either at the dentine surface or inside the tubules were imaged together with any other features representative of the individual sample.

All of the fractured SEM dentine samples were imaged and 10 representative images, 5 treated and 5 untreated were prepared for scoring of occlusion by 3 independent examiners. They scored the tubules as either 1 (occluded), 2 (unoccluded) or 3 (non-evaluable). The information bar from the SEM images was removed and each sample was assigned a randomised number so that the examiners were blind as to which sample had received treatment or not.

3.3.13 Statistical analysis

The statistical test used on the results obtained from the flow cell model was the Wilcoxon Signed Rank Test with analysis of covariance for split mouth, placebo-controlled design. Due to the wide range in standard deviation the data was put into log before being transferred back to the original scale before the analysis of covariance test was performed. Random effects were included in the covariance structure of the model to account for the within subject variability. Statistical comparisons utilised two-sided testing with a 5% significance level. Any significant difference between baseline and post treatment with potassium oxalate was identified as well as any difference between the treated and non-treated flow rate following the in situ phase of the study. The analysis was performed by a professional statistician employed by Procter and Gamble.

3.4 Results

3.4.1 Participant and sample through flow

The study commenced on 19th September 2016 and the last participant completed on the 21st October 2016. A total of 20 participants were recruited to take part in the study and all 20 participants completed the full duration. Subjects ranged in age from 21 to 61 with an average of 38 years. 17 subjects were female and 3 were male.

There were 0 screening failures and 0 baseline failures. No participants withdrew or were lost to follow-up on the study. All the participants were satisfactorily compliant with the study protocols.

No observed and/or reported evidence of any hard or soft tissue damage associated with the use of the test product was reported and there were 0 adverse events reported for the study.

A total of 40 dentine disc samples were sectioned from 40 separate whole human 3rd molar teeth for use in this study. Great care was taken to choose teeth that were both large enough to provide enough diameter for the dentine disc to seal into the flow cell as well as avoiding teeth where the enamel was visibly cracked after extraction as this would lead to a weakened enamel ring surrounding the dentine and an increased chance of leakage once placed into the flow cell. If the pulpal horns could be found in the dentine disc or if the outer enamel appeared cracked the sample was rejected resulting in a 1 in 4 failure rate, leading to 50 teeth being sectioned in order to produce the 40 required samples.

3.4.2 Hydrodynamic flow rates

The flow rate measurement of HS through the dentine discs was repeated 4 times for each sample at baseline, post-treatment and post-appliance. The in-situ experiment used a total of 40 samples, providing a much larger data set than the developmental experiments described in section 2.4. A variation in baseline flow rates was also noted in this experiment for some of the samples however there was no clear pattern in which of the 4 readings differed. Some samples such as sample 27 maintained baseline flow rates within a 2% margin of each measurement. However, with sample 18 the baseline flow rates fell within a maximum 45% margin of each measurement. Ideally a sample such as this would have been rejected and replaced with a sample that achieved more cohesive baseline flow rates. Unfortunately, the amount of human tissue available with the correct sample diameter was limited and the timeframe for the investigation did not allow for further tissue to be sourced.

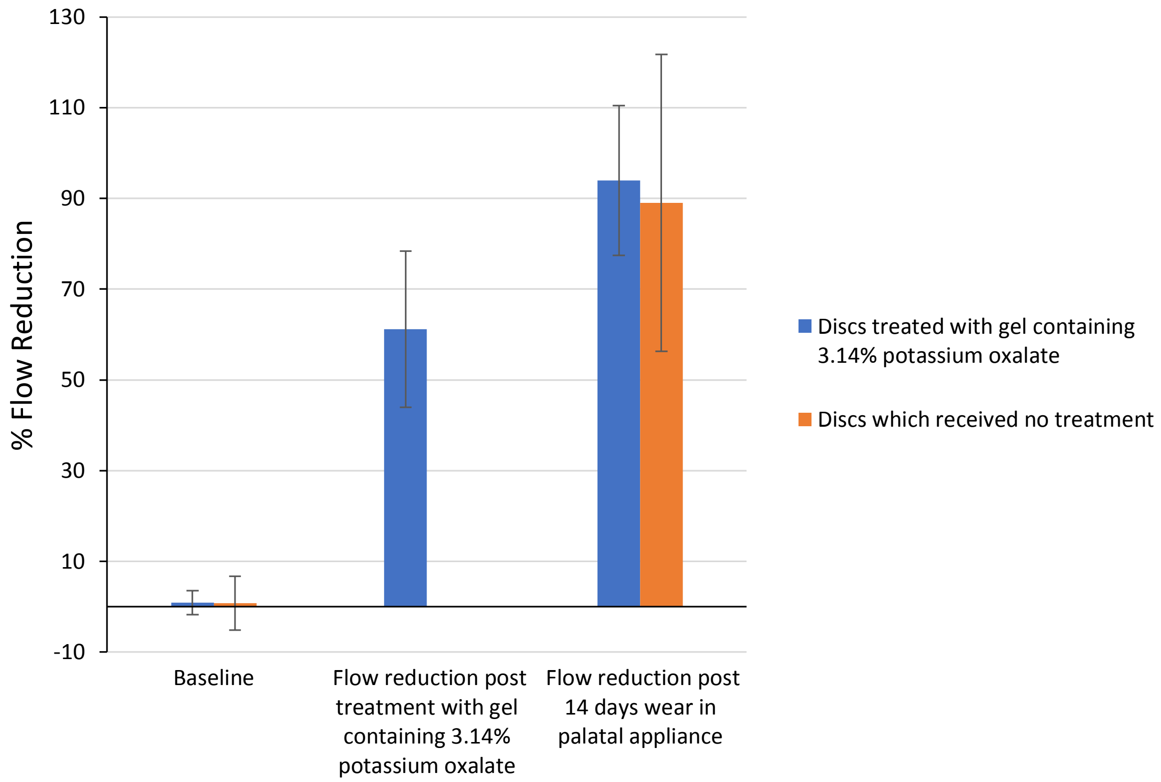


Figure 3.3 Hydrodynamic flow rates through dentine for all samples expressed as a % of baseline flow rates. For samples treated with gel containing 3.14% potassium oxalate the flow reduction for both post treatment and post 14 days wear in appliance was individually calculated from baseline. For discs which received no treatment flow reduction post treatment data was not collected and post 14 days wear reduction was calculated from baseline.

The data presented for this study has been expressed as a median percentage flow rate change from baseline for each of the flow rate measurements taken, as seen above in figure 3.3. The data is presented as a percentage flow rate because there was a wide range of flow rates recorded, therefore analysing them in this way allows for clearer comparisons to be made. Each sample acted as its own control between baseline and post-treatment measurements which is important due to the individual morphology of each dentine section being different. A full copy of flow rate per minute data and the % flow rate from baseline data can be found in appendix III and IV.

One of the treated samples broke during removal from the appliance and therefore no post-appliance data is available for this individual sample. The median values presented in Figure 3.3 have been calculated after 3 treated samples and 3 un-treated samples were removed from the data set as these samples were considered outliers which is expanded upon in the discussion below.

The samples treated with the potassium oxalate strip (n=17) reported a 61.2% reduction in flow rate post-treatment, reducing the flow rate from 90.3 µl/min at baseline to 36.7 µl/min which was highly significant (Wilcoxon Signed Rank Test, $p<0.0001$). Following the housing of samples (n=16) in the appliance worn in the oral environment the samples that were treated with the potassium oxalate strip attained a total reduction in flow rate of 93.8% from baseline flow rate which gave an average flow rate of 9.6 µl/min .

The control samples received no treatment so there is no recorded value for the post-treatment measurement. After removal from the appliance, the control samples (n=17) showed a reduction in flow rate of 89.1% from the baseline value. The amount of liquid passing through the samples reduced from 123.8 µl/min at baseline to 40 µl/min after being worn in the oral environment which was also highly significant (Wilcoxon Signed Rank Test, $p<0.0001$).

Following exposure to the oral environment, flow rates between the two groups were compared using analysis of covariance. The samples treated with the potassium oxalate containing strip attained a statistically significantly greater reduction in flow rate when compared to the non-treated samples ($p=0.0038$).

3.4.3 Dentine disc imaging using FIB-SEM and EDX analysis

Representative FIB-SEM images of samples treated with a gel containing 3.14% potassium oxalate and housed in a palatal appliance for 14 days are shown in figures 3.4, 3.6 and 3.7. The sample depicted in figure 3.5 was housed in the palatal appliance for 14 days but received no prior treatment.

The milling process implemented by the FIB-SEM removed material at a 52° angle in relation to the dentine disc surface. Through careful site selection the maximum number of dentine tubules were uncovered to increase the possibility of exposing formations within the tubules. However, it was not possible to predict the course of the dentine tubule and once the initial 2-3 µm of material had been removed from the dentine surface, often the tubule orientation would ‘snake’ away from the ion beam that exposed the tubule, leading to inconsistency in the area of tubule that could be examined. The width of the trench cut into the sample was a major factor in the amount of time taken to complete tubule exposure. Figures 3.1 to 3.4 exhibit the width of trenches ranging between 15 µm to 40 µm.

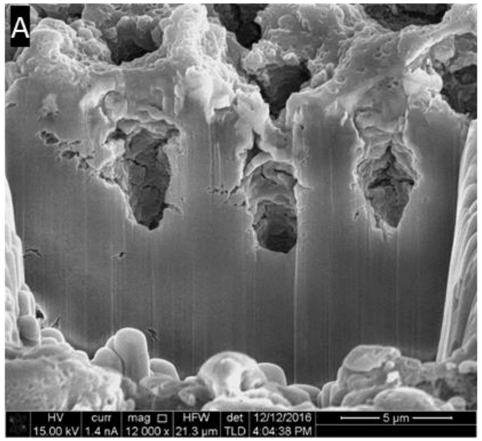
Figure 3.4 A shows a representative image of a treated sample where the FIB failed to penetrate far enough down the tubule length, exposing only 5 µm of the tubule from the dentine surface. If further material had been removed, the top section of the tubule would have been lost as the tubule was beginning to orientate away from the exposed area due to the morphology of the tubule.

The image in Figure 3.4 B was taken from a different site on the same sample and was able to penetrate up to 12 µm into the tubule. The tubule openings appear to be patent however there is possibly some precipitation of calcium oxalate crystalline strictures observed within the tubules but they do not span the entire width. An EDX analysis was performed at 4 sites across this sample and recorded the highest percentage of calcium levels found in any of the samples analysed. As reported in table 3.1, site 1 exhibited 79.33% calcium and site 2 inside the sample dentine tubule recorded 64.95% calcium. This is 30-40% higher than other sites in treated samples and it perhaps indicative of calcium oxalate crystal formation occurring. Also, site 2 reported similar levels of calcium at 65.84%, perhaps suggesting calcium oxalate formation in this neighbouring tubule. Only at site 4 were the calcium levels in line with those found in other treated samples at 40.16% where an increased level of oxygen was recorded.

Figure 3.5 A and B display tubules from a sample which received no treatment. The maximum depth of tubule exposure is around 10 µm at site 1 in image B. The tubules appear to be open throughout the full length but are orientating away from the trench. There appears to be a layer of debris covering the openings of the tubules however this is somewhat exaggerated by the angle the image is taken from. As expected the chemical analysis reported in table 3.2 showed almost identical findings between areas within the tubules at sites 1&2 and outside of the tubule walls at site 3 with the level of calcium around 45% at all sites.

Figure 3.6 A and B show images of a treated specimen. The FIB-SEM images are taken from the same site however the trench was extended to include further tubules in image B. The openings to the tubules appear patent and the dentine surface appears to be clear of debris. Both images may display significant precipitation of oxalate crystals at several sites inside the tubules. Multiple crystal structures appear to join and occlude the full diameter of tubule lumen in 4 of the tubules displayed in image B. The deposits can be noted between 5 – 15 µm in depth into the tubule. EDX analysis was performed at 5 sites across the sample. Site 3 reported calcium levels at 61.78% which was around 20% higher than at any other site and a possible indication of the formation of calcium oxalate crystals. The site also reported the lowest level of carbon at 5.28%. Site 2 was positioned on the intertubular dentine and reported lower levels of calcium than the other sites at 23.8%, not including site 4 which was positioned outside of the tubules in an attempt to identify a piece of surface debris, reporting only 14.84% calcium. Sites 2 and 4 reported increased levels of oxygen for this sample at around 30%, however the level is consistent with other samples tested. Both gallium and a trace amount of chlorine are listed in the EDX results however this is a result of the sample coating and gas in the chamber and therefore not related to the dentine structure.

Figure 3.7 A and B show images of a treated specimen. The FIB-SEM images were taken from the same site at 2 different stages of the FIB milling process. The sample surface appears to be clear of significant debris and the tubules patent. The tubules have been exposed to a maximum milling depth of 20 µm and appear to be completely unoccluded suggesting no penetration of the treatment product at this specific site. The rod structures seen in the foreground are an artefact of the milling process and do not relate to the treatment. The EDX chemical analysis was performed at 2 sites, on the intertubular dentine and inside a tubule with both sites reporting almost identical chemical profiles.



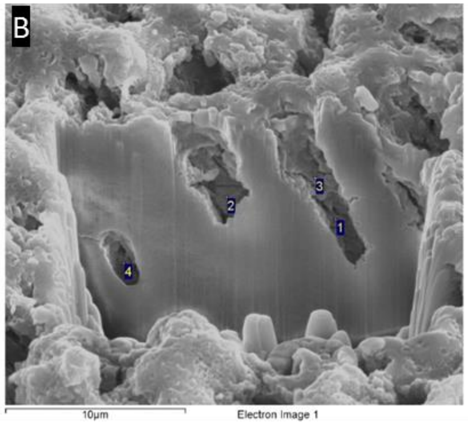
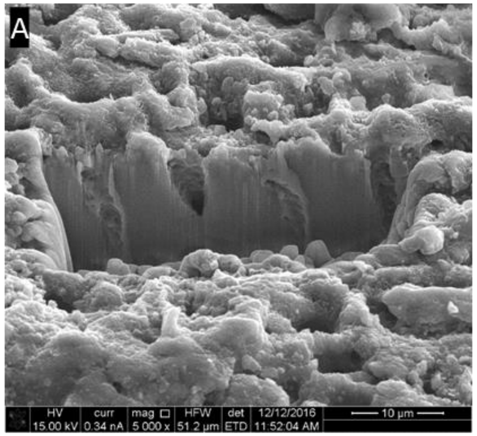


Figure 3.4: A) FIB-SEM image showing a cross section through dentine tubules from sample 15 treated with gel containing 3.14% potassium oxalate B) SEM image of a different section from sample 15, the image is labelled with location of EDX analysis sites 1, 2, 3, and 4 corresponding to Table 3.1.

Table 3.1 EDX analysis showing chemical composition of sites labelled in figure 3.4 B. All results presented in weight %

Spectrum	C	O	F	P	Ca	Total
1	1.24	2.44		17.10	79.22	100.00
2	2.43	7.29		24.44	65.84	100.00
3	3.01	5.72		26.33	64.95	100.00
4	5.05	33.92	1.22	19.64	40.16	100.00



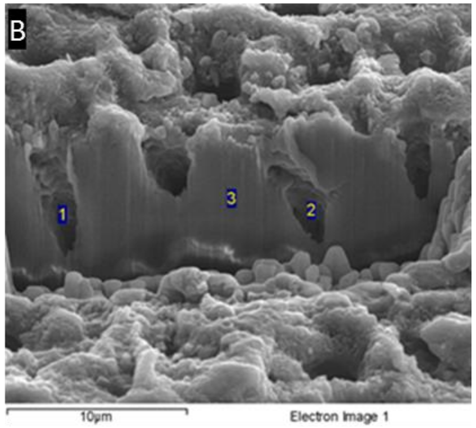


Figure 3.5:A) FIB-SEM image showing a cross section through dentine tubules from sample 6 which received no treatment B) SEM image of sample 6 showing labels for the locations of EDX analysis sites 1, 2 and 3 which correspond to table 3.2

Table 3.2 EDX analysis showing chemical composition of sites labelled in figure 3.5 B. All results presented in weight %

Spectrum	C	O	Mg	P	Ca	Total
1	5.19	29.98	0.28	21.60	42.95	100.00
2	3.90	29.58	0.26	20.79	45.47	100.00
3	3.84	22.99	0.31	23.15	49.70	100.00

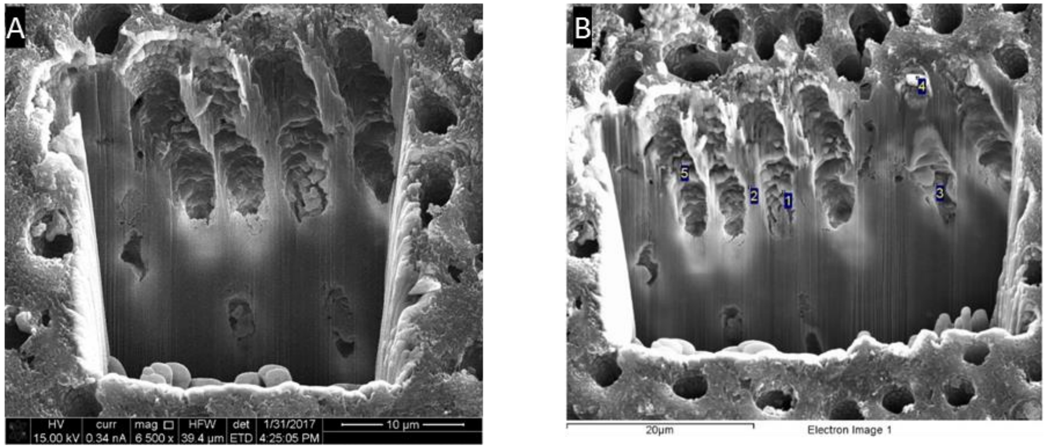


Figure 3.6: A) FIB-SEM image showing a cross section through dentine tubules from sample 21 which had been treated with gel containing 3.14% potassium oxalate B) SEM image of sample 21 showing the locations of EDX analysis sites 1,2,3,4 and 5 which correspond to table 3.3.

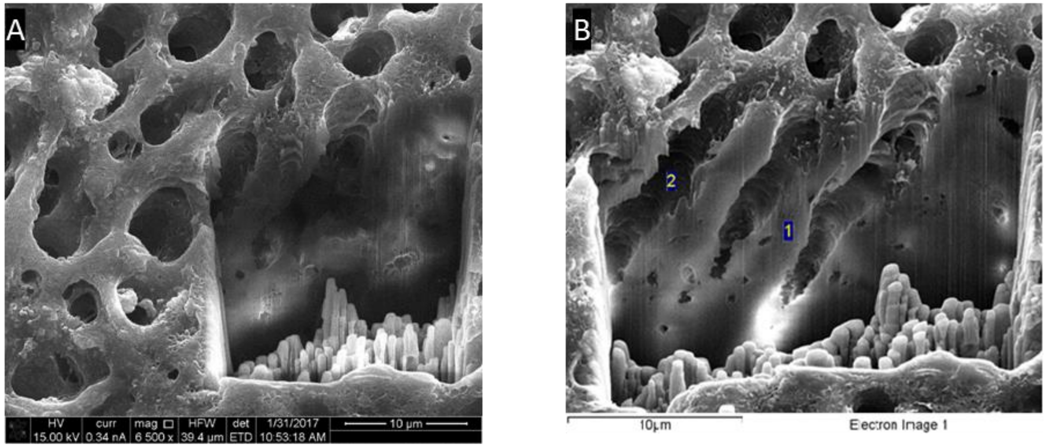


Figure 3.7: A) FIB-SEM image of dentine tubule cross section from sample 3 treated with gel containing 3.14% potassium oxalate B) SEM image of dentine tubule cross section from sample 3 treated with gel containing 3.14% potassium oxalate, the image is labelled with locations of EDX analysis sites 1 and 2.

Table 3.3 EDX analysis showing chemical composition of sites labelled in figure 3.6 B. All results in weight %.

Spectrum	C	O	P	Cl	Ca	Ga	Total
1	8.29	15.67	18.77	0.59	42.08	14.61	100.00
2	22.67	28.69	11.31	0.41	23.80	13.13	100.00
3	5.28	8.77	18.16	0.57	61.78	5.44	100.00
4	27.84	30.58	6.89	0.45	14.84	13.34	100.00
5	12.04	16.33	13.20	0.95	33.43	24.06	100.00

Table 3.4 EDX analysis showing chemical composition of sites labelled in figure 3.7 B. All results in weight %

Spectrum	C	O	P	Cl	Ca	Ga	Total
1	2.75	22.37	22.52	0.41	48.20	3.76	100.00
2	3.64	28.32	19.34	0.40	40.38	7.93	100.00

3.4.4 Imaging of fractured dentine discs

Following 14 days exposure to the oral environment the dentine discs were fractured so that SEM analysis of cross sections of the samples could be taken. Figure 3.8 show samples that had not been treated with potassium oxalate whilst Figure 3.9 show samples that had been treated with a gel containing 3.14% potassium oxalate. All samples had been housed in a palatal appliance for 14 days.

The methodology of fracturing the dentine disc samples was successful in providing a means to examine the lumen of dentine tubules. The technique produced a central fracture across the dentine disc in the majority of cases although some samples failed to fracture with the first impact, fractured into more than 2 pieces or did not fracture centrally. Where this occurred the sample was re-fractured.

3.4.4.1 Cross sectional images of untreated samples

The images presented below in Figure 3.8 depict the longitudinal view of fractured dentinal tubules following the in situ phase of the study from samples which received no treatment. The coronal side of the dentine disc which was exposed to the oral environment is visible at the top of the images. The tubules remain unoccluded as indicated by red arrows, yet the tubule lumen in Figure 3.8 E appears to have narrowed substantially in comparison to other visible tubules which could indicate penetration of debris from the oral environment although this is an isolated finding.

In Figures 3.8 A, B and D the dentine surface appears rough but free from debris. An indication of surface debris can be noted in Figures 3.8 C and E. A homogenous coating can be seen occluding the openings to several dentine tubules at the locations labelled with green arrows however other visible tubules appear to remain open.

3.4.4.2 Cross sectional images of samples treated with gel containing 3.14% potassium oxalate

The images presented below in Figure 3.9 depict the longitudinal view of fractured dentinal tubules following the in situ phase of the study from samples which were treated with gel containing 3.14% potassium oxalate. The coronal side of the dentine disc which was exposed to the oral environment is visible at the top of the images.

Multiple dentine tubules can be seen to contain crystalline deposits extending to a depth of between 5 to 25 µm. The fragments labelled with the red arrows are indicative of a calcium oxalate crystal which fully occludes the entire diameter of the tubule lumen, other such fragments can be seen throughout the images. The clearest examples of these formations can be noted in Figures 3.9 C and D. The crystal dimensions vary, some include crystals that occlude a large portion of the tubule diameter whilst there are also smaller bunches of crystals that join to occlude the tubule which indicate calcium oxalate crystals.

The tubule labelled with the yellow arrow in Figure 3.9 B appears to contain a fragment which fills the entire tubule lumen but the structure of the blockage is not crystalline, it can also be found in other tubules. The dentine surface in the same image is covered in a dark uniform detritus which is remnants from mistakenly mounting the sample working surface in the wrong orientation onto a carbon tab as part of the FIB SEM preparation and therefore would not have been present when flow rate measurements were taken.

The dentine surface in Figures 3.9 A, C, D and E appear to be clear of any debris and the tubule openings are patent.

Table 3.5 below displays the scores assigned to each image by the examiners. The examiners were unanimous in their scoring of the images. Samples 6, 8, 10, 24 and 28 were samples which received no treatment prior to housing in the palatal appliance for the in-situ phase and all 3 examiners scored the images as 2 which was the code for tubules being unoccluded. Samples 1, 3, 5, 15 and 33 were samples that had been treated with gel containing 3.14% potassium oxalate prior to the in-situ phase and all 3 examiners scored the images as 1 which was the code for tubules that showed a degree of occlusion. None of the images received a 0 (not evaluable) score.

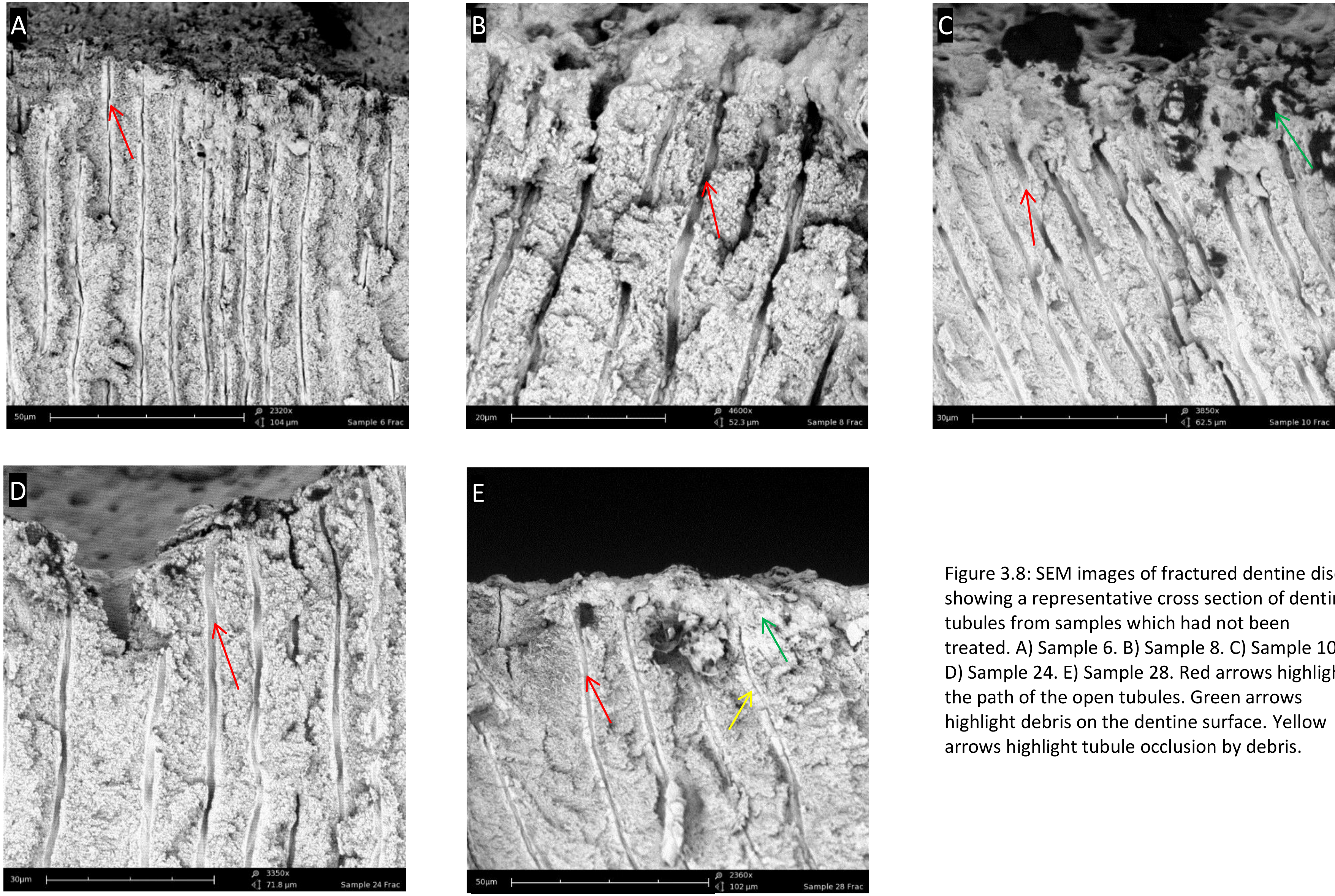


Figure 3.8: SEM images of fractured dentine discs showing a representative cross section of dentine tubules from samples which had not been treated. A) Sample 6. B) Sample 8. C) Sample 10. D) Sample 24. E) Sample 28. Red arrows highlight the path of the open tubules. Green arrows highlight debris on the dentine surface. Yellow arrows highlight tubule occlusion by debris.

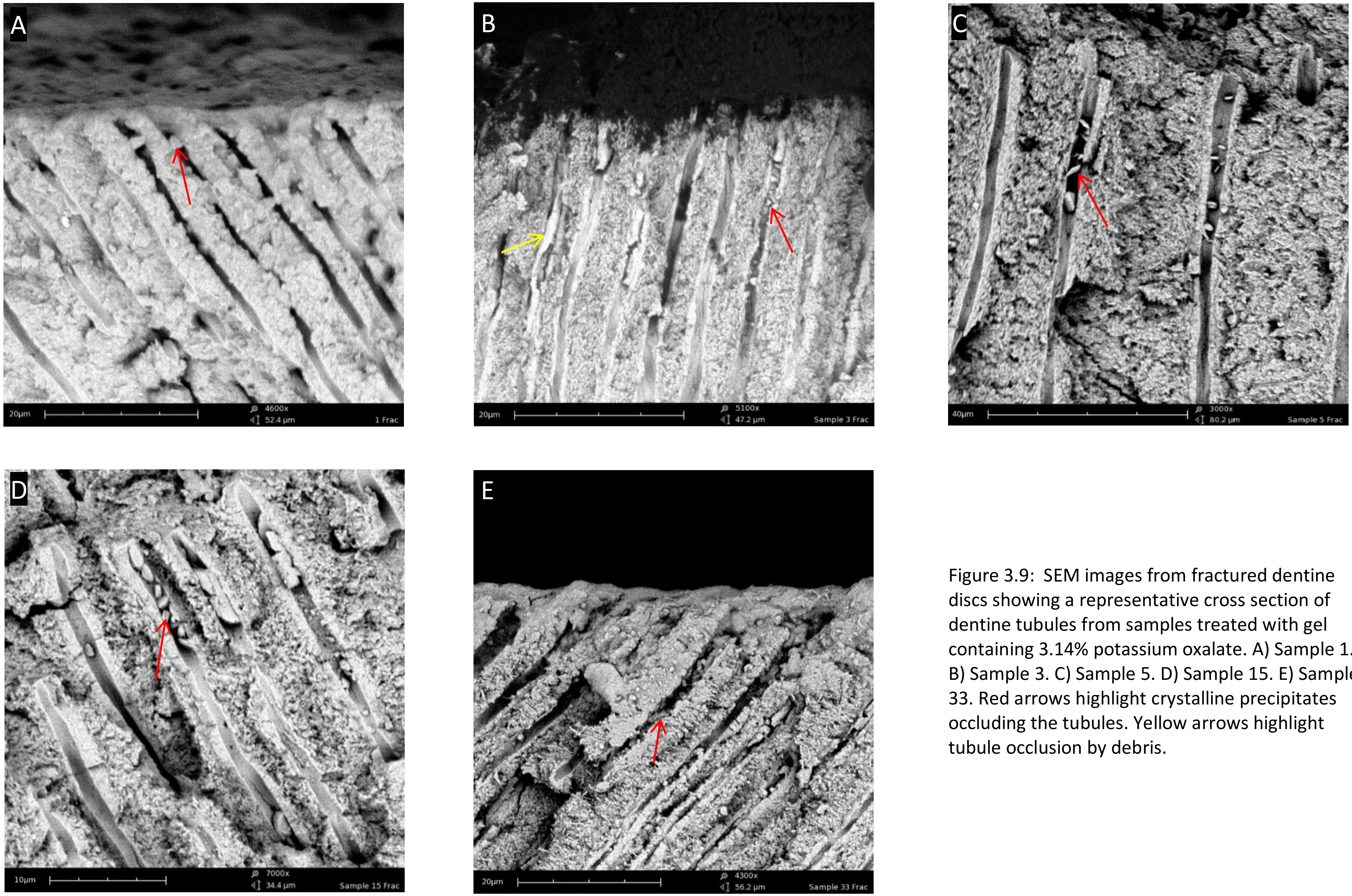


Figure 3.9: SEM images from fractured dentine discs showing a representative cross section of dentine tubules from samples treated with gel containing 3.14% potassium oxalate. A) Sample 1. B) Sample 3. C) Sample 5. D) Sample 15. E) Sample 33. Red arrows highlight crystalline precipitates occluding the tubules. Yellow arrows highlight tubule occlusion by debris.

Examiner Grading					
Image Number	Sample Number	Sample Treatment	Examiner 1	Examiner 2	Examiner 3
1	24	No treatment	2	2	2
2	28	No treatment	2	2	2
3	3	Treated	1	1	1
4	6	No treatment	2	2	2
5	5	Treated	1	1	1
6	8	No treatment	2	2	2
7	10	No treatment	2	2	2
8	15	Treated	1	1	1
9	33	Treated	1	1	1
10	1	Treated	1	1	1

Table 3.5: Examiners scores of representative SEM images for levels of tubule occlusion in fractured dentine discs, 5 samples were treated with gel containing 3.14% potassium oxalate and 5 samples received no treatment. 0 = Not evaluable; 1 = Occluded; 2= Unoccluded. Samples 6, 8, 10, 24 and 28 received no treatment. Samples 1, 3, 5, 15 and 33 were treated with gel containing 3.14% potassium oxalate.

3.4.5 Surface debris on dentine discs

The results from the flow cell model reported a significant reduction in fluid flow rate following exposure to the oral environment for dentine samples that had not received any treatment. This finding warranted further investigation as it could provide valuable information regarding the effect of the in-situ phase of the study.

After the samples had been removed from the appliance it was noticed that many had changed colour from the natural state shown in figure 2.2 and in some cases surface debris was clearly visible by the naked eye. When placed under SEM clear evidence of surface debris could be noted on some of the samples. A selection of representative images are presented below in Figure 3.10. To accompany the SEM images, a photograph of each corresponding fractured dentine disc is presented in Figure 3.11. The photographs have been taken from a side on angle and are representative of the overall colour and debris found on the dentine.

Figure 3.10 A depicts a top down view of the dentine surface from a sample which received no treatment, the image was taken on the FIB-SEM before milling a trench. The dentine tubules appear to be partially occluded by small particles of debris throughout the image. The yellow arrow points toward a piece of debris which covers the majority of a tubule opening. The green arrow marks a smaller blockage which is partly covering the tubule. The corresponding photograph for this sample in figure 3.11 A displays patches of darker areas running across the surface next to the more typical yellow dentine colour. Figure 3.10 B depicts a longitudinal view of dentine tubules in a fractured dentine disc from a sample which received no treatment. A homogenous coating of material can be seen occluding many of the dentine tubules such as the one highlighted by the yellow arrow. This coating appears to have large contrasts suggesting different types of deposit across the dentine surface, signposted by a green arrow. The photograph of this sample is shown in figure 3.11 B and presents a direct correlation with the SEM image. A large black area of material can be seen attached to the sample. The observable dentine has become grey in colour and the enamel surrounding the outer rim of the fractured disc is verging on black. Figure 3.10 C depicts a longitudinal view of dentine tubules in a fractured dentine disc from a sample which received treatment with gel containing 3.14% potassium oxalate. The surface is entirely covered by a coating of material which appears homogeneous and occludes the dentine tubule openings as highlighted by the yellow arrow. The corresponding dentine sample in the photograph in figure 3.11 C appears to have become a dark grey colour in the central section and at the periphery. Small black areas of debris can also be seen on the dentine surface in several locations.

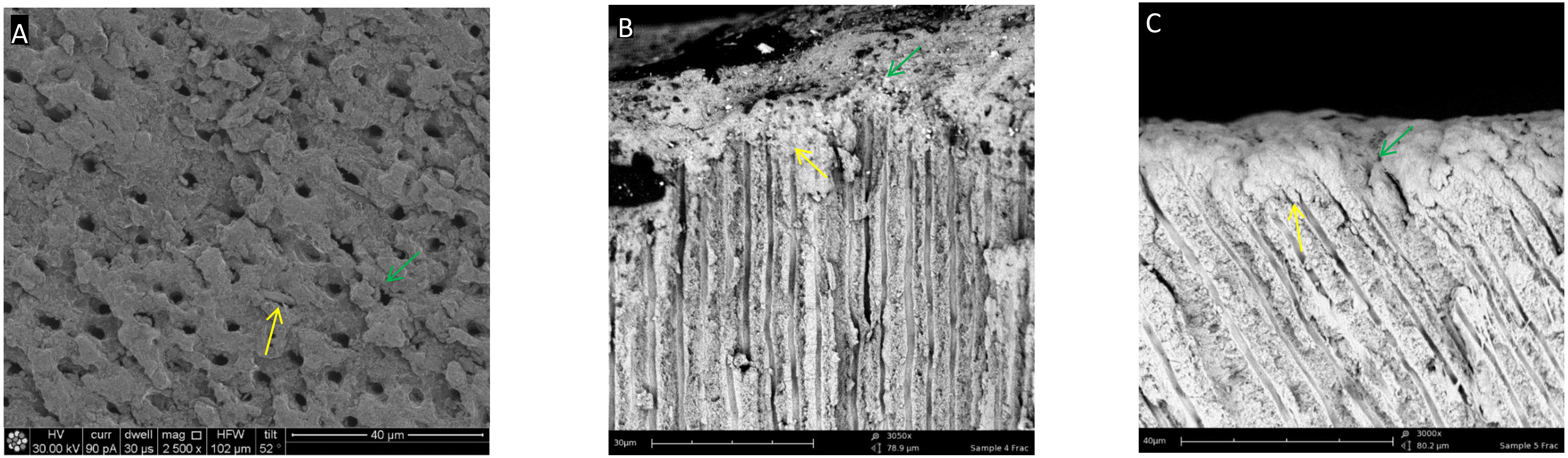


Figure 3.10: A) FIB-SEM top down image of dentine surface and debris coating on sample 6 which received no treatment B) SEM image of cross sections of dentine tubules showing debris coating the dentine surface from sample 4 which received no treatment C) SEM image of cross sections of dentine tubules showing debris coating the dentine surface from sample 5 which received treatment with gel containing 3.14% potassium oxalate. Green arrows highlight debris on the dentine surface. Yellow arrows highlight debris penetrating into the dentine tubule.

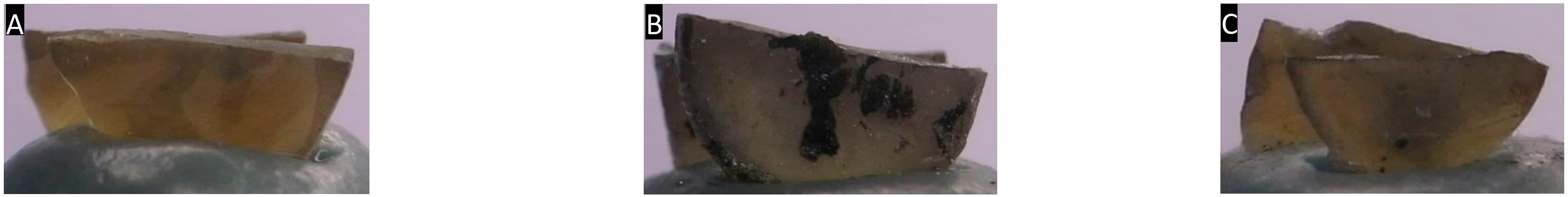


Figure 3.11: A) Side on photograph of fractured dentine disc showing discolouration and debris coating on sample 6 which received no treatment B) Side on photograph of fractured dentine disc showing discolouration and debris coating on sample 4 which received no treatment C) Side on photograph of fractured dentine disc showing discolouration and debris coating on sample 5 which received treatment with gel containing 3.14% potassium oxalate.

3.5 Discussion

There were three objectives to the work presented in this chapter. Firstly, to test the effectiveness of a potassium oxalate containing strip in reducing the rate of fluid flow through dentine disc samples compared to the baseline rate.

The second objective was to test the suitability of the flow cell model in providing quantifiable data relating to the resilience of the treatment applied to a dentine disc after an in situ phase. The dentine disc model has already proved valuable in initial in vitro screening of potential desensitising agents for the relief of the symptoms associated with DH (Ling *et al.*, 1997; Mordan *et al.*, 1997; Hiller *et al.*, 2018). This work has taken this model further to determine its effectiveness in showing durability of a test product following an in situ phase.

The final objective was to obtain micrographs of the dentine tubules using FIB-SEM with EDX chemical mapping and SEM in order to provide a source of data to support the findings of the flow cell model. For the purposes of corroborating the flow cell data the SEM images in this study are invaluable.

The hydraulic conductance results from this study show that the 3.14% potassium oxalate treatment applied to dentine discs was successful in reducing the hydraulic conductance of fluid through the dentine tubules by 61.2%. The treatment was applied to the dentine discs for 10 minutes, immediately after taking baseline flow rate measurements. When potassium oxalate is exposed to the dentine surface it forms a chemical reaction which allows ion exchange between available calcium from the dentine surface or dentinal fluid or in the case of this study the participants own saliva and Hartmann's solution to exchange with potassium to form partially soluble calcium oxalate crystals (Varoni *et al.*, 2017). The single exposure of 10 minutes in this study suggests that the calcium oxalate crystal formation was rapid and only one application of the product was necessary to statistically significantly reduce fluid flow. This result is in agreement with the findings of Hare et al (2016b) who reported an even greater decrease in fluid flow after treatment with potassium oxalate with a 70% reduction in a similar in-vitro experiment. It is interesting to note that this study included an additional stage whereby the treated side of the dentine disc was flushed with artificial pulpal fluid, consisting of 1.2% bovine serum albumin diluted into HS. It is possible that this would increase the formation of calcium oxalate crystals in the dentine tubules due to the greater availability of calcium ions from the HS, as it was applied to the disc from both the treated and un-treated sides. In the present study, the HS originated from the un-treated side only and it was not possible to include bovine serum on the treated surface due to the ethical implications involved with the introduction of participants housing the dentine discs at the in situ phase, therefore the additional flushing was not included. It was hypothesised that the use of the participants own saliva in sample preperation and HS running though the dentine would be sufficient for enough calcium ion exchange to take place.

The adaption of the Pashley model has been used elsewhere to test the ability of oxalate derivatives to reduce hydraulic conductance through tubule occlusion. In the work of Santiago et al (2006) both 3% and 6% monohydrate potassium oxalate gels were investigated for occlusion potential on dentine discs. This study reported an average dentine permeability decrease of 75% for samples in the treated groups immediately after application. Remarkably, this reduction was seen to fall to below 20% when re-testing the samples 15 minutes after the treatment was applied and stayed roughly at this level even after the introduction of a 6% citric acid challenge 30 minutes after the initial treatment. It should be noted that this study applied a 69 kPa pressure to the deionised water permeating through the samples under investigation, this is in contrast to the HS under 207 kPa used in the present study. Santiago et al (2006) hypothesised that the passive increase in fluid flow was as a result of time passing and that increased intratubular resistance may occur under higher pressures as used in this present study. Under higher pressure, any internal tubule contents may be compressed against the tubule walls, aiding in blocking the tubules. In the present study it is possible that this compression effect contributed to the reduction in permeability found following treatment, however it is not possible to say to what extent without further testing. Conversely, evidence also suggests tubular contents are easily removed by fluid filtration through dentine at high pressure (Camps *et al.*, 1997). The present study employed a pressure of 207 kPa as it has been found to report moderate pain in vivo (Ahlquist *et al.*, 1994) and therefore can be advocated as an appropriate pressure for testing of the occluding agent in this study. The work of Santiago et al (2006) did not employ a sample brushing process with human saliva prior to treatment and the solution under pressure was DI water not HS which contains an increased level of calcium in comparison. These factors may be the reason for the increase in permeability after 15 minutes that they saw as there may be limited available calcium for the potassium ions to exchange with. In the present study the samples were not tested again after treatment until the end of the in situ phase 14 days later, therefore accurate connections cannot be made.

In a more recent study (Varoni *et al.*, 2017) the reduction in permeability of dentine discs treated with a potassium oxalate hydrogel reached a 69% reduction after 10 minutes of treatment. This result is comparable to the 61% reduction in flow rate established in the present study. It should also be noted that dentine samples were etched with ortho-phosphoric acid prior to treatment which evidence suggests may be more effective at removing the smear layer and opening the tubules than the citric acid used for the present study (Bagmar *et al.*, 2013). It could be hypothesised that this further removal of smear layer allowed for greater penetration of the potassium oxalate hydrogel into the dentine tubules therefore further decreasing the permeability.

Measuring the hydraulic conductance of dentine samples has also been used to investigate other occluding agents found in toothpaste where their mode of action is to form deposits across the dentine surface or in the tubules in order to reduce fluid flow. A study investigating the effect of arginine calcium carbonate in toothpaste has shown that the permeability of the dentine after a single treatment was reduced by 63% and reduced further to 82% after 14 treatments which is comparable to the results observed for a single treatment in this study. SEM images confirmed a surface coating was visible at the dentine surface and showed penetration into the tubules (Petrou *et al.*, 2009). Furthermore, it has been demonstrated that occlusion with arginine calcium carbonate is resistant to various acid challenges in vitro (Patel *et al.*, 2011b).

In a study carried out by Wang *et al.* (2010) an active bioglass containing toothpaste was investigated for its ability to reduce fluid flow through dentine samples as represented by the movement of an air bubble through a micro-capillary tube. Active bioglass consists of calcium sodium phosphorus and silica which releases deposits of crystalline hydroxyl-carbonate apatite (Golpayegani *et al.*, 2012). The reduction in dentine permeability identified by Wang et al (2010) was shown to be 81.5% from that of baseline measurements after a single 2 minutes of brushing with the bioglass containing toothpaste. This result shows a high level of occlusion after only 2 minutes but it is worth noting that in this work treatment with deionised water alone reduced the fluid flow by 70 %. It is suggested that the application of brushing the paste on the surface caused the formation of a new smear layer that occluded the tubules. The application of the potassium oxalate onto the dentine surface using a gel strip rather than brushing the surface avoided the formation of a smear layer on the surface in this study.

It has also been proposed that deposition of strontium acetate within the dentine tubules will occlude the tubule lumen to reduce permeability. However, Patel et al (2011a) found that after one brushing treatment with a toothpaste containing strontium acetate the reduction in fluid flow was 27% and therefore not as successful as many other occluding agents.

A further toothpaste ingredient investigated for its ability to occlude dentine tubules is calcium phosphate. A reduction in hydraulic conductance of 92% was reported immediately after treatment with an adhesive agent containing calcium phosphate (Thanatvarakorn *et al.*, 2013), however this is a professionally administered agent in contrast to the potassium oxalate strips used for this investigation.

In the present study, following the insertion of the dentine discs into the palatal oral appliance worn by study participants for 14 consecutive days, the dentine discs were removed and their hydraulic conductance re-tested. The samples which had been treated with potassium oxalate reduced the fluid flow by 93.8% from that of the original baseline flow rate. This remarkable further reduction in hydraulic conductance following the in situ stage was unexpected but there are some potential explanations. Firstly, it is possible that following the post-treatment measurements the formation of calcium oxalate crystals increased during the in situ phase due to deposits of the potassium oxalate gel remaining in the porous dentine and further availability of calcium from the oral environment. However, this seems unlikely considering the samples were rinsed with DI water immediately following treatment and the combination of the surface brushing between measurement cycles together with the pressure the dentine was under during the post treatment flow rate measurements would leave little chance of substantial gel deposits remaining.

The control samples in this study, which received no treatment prior to insertion into the oral appliance, also reduced the hydraulic conductance of fluid passing through the dentine by 89.1% from the baseline flow rates following removal from the appliance. The decrease in flow rate was evidenced for both sample groups after exposure to the oral environment. The most likely scenario as to why the flow rate was reduced further is the formation of a smear layer across the dentine surface, acting to further occlude the dentine tubules from the post-baseline and post-treatment levels. It has been suggested that the presence of a smear layer is the most effective condition to obstruct dentine tubules and reduce liquid through flow (Santiago *et al.*, 2006). In vivo, the dentine at the cervical area is naturally covered by cementum. If recession occurs through causes such as overly enthusiastic tooth brushing or periodontal disease the cementum is rapidly lost and will never again cover the dentine following the recession (Bevenius *et al.*, 1994). Exposed dentine is also believed to be covered by a smear layer covering the tubules with calcium phosphate deposits originating from saliva (Addy, 2002). This layer could be removed by physical or chemical agents which open the dentinal tubules. It is therefore possible that this smear layer formed on the dentine disc during the in situ phase of the present study. The cleaning regime in place for the care of the palatal appliance specifically avoided physical contact by the toothbrush with the samples to protect the delicate dentine. Consequently, there would have been minimal opportunity for removal of any smear layer present as a result of immersion in saliva.

The hydraulic conductance of the dentine discs investigated in this study can be seen to have been affected by the in situ phase to a certain degree regardless of whether they received treatment with potassium oxalate or not. Where a statistical comparison is made between the samples that had been treated with gel containing 3.14% potassium oxalate and samples which received no treatment, the samples that had been treated reported a statistically significant reduction in flow rate compared to the untreated samples. This model was therefore effective in showing the occluding potential of potassium oxalate containing strips in reducing fluid flow through dentinal tubules following treatment, as well as following 14 days of exposure to the oral environment. Despite showing a reduction in fluid flow for samples that had been treated and untreated after removal from the oral appliance, the treated samples maintained a superior reduction in hydraulic conductance.

Once all the data had been collected from the flow cell, the values for 4 treated samples and 3 untreated samples were removed from the statistical calculations presented in this study. The flow data from these samples were considered outliers for either being extremely slow or fast in the time it took the air bubble to advance in comparison to the baseline measurements taken from the same sample. The possible reasons for these extremes in flow rates were that either a sample had become coated in some food debris that was also visible by eye, resulting in exceptionally slow flow rates, or a sample had developed a hole in the dentine or a crack in the surrounding enamel, in which case the flow rate was exceptionally fast, too fast to take a flow rate measurement accurately by eye. This could have been due to damage sustained whilst housed in the oral appliance, possibly as a result of hard food consumption or the samples being damaged during removal from the oral appliance after the in situ phase despite great care being taken. It should also be noted that 1 of the treated samples did break into pieces following removal from the appliance and therefore it was not possible to mount the dentine disc into the flow cell apparatus for a post-appliance measurement.

The development of the hydrodynamic theory by Brännström and his colleagues was conducted through a series of in vitro experiments involving the application of stimuli to exposed dentine inducing fluid flow through the tubules. In one experiment single rooted teeth freshly extracted were connected to a transducer, amplifier and recorder by a polyvinyl tube filled with saline covering the root of the tooth (Brannstrom *et al.*, 1967). Similarities can be seen between these studies and the present study methodology. However, one of the most fundamental differences is that the present study induced the fluid flow through the dentine using a pressurised system. The work of Pashley and his collaborators can be seen to build upon many of the ideas proposed by Brännström. The original Pashley model on which the present studies apparatus is based upon consists of a modified pressure cooker containing saline solution and linked to a nitrogen cannister pressurising the liquid to 103 kPa (Pashley and Galloway, 1985a). A dentine disc was situated between two rubber washers and mounted into a custom plastic housing. This housing was connected to the pressurised liquid container by tubing which was intersected by a micro syringe part way allowing for insertion of an air bubble into the system. The permeability of the dentine was calculated by recording the progress of the air bubble across a micropipette and converting this time measurement into hydraulic conductance.

In the present study several modifications have been made to the system used although it is principally alike. The primary change being that the Hartmann's solution was pressurised with compressed air at 207 kPa when the flow measurements were taken, a pressure gradient reported to produce moderate levels of sensitivity in vivo. The pressure used when treatment was applied to the dentine sample was 3 kPa being consummate with resting in vivo pulpal pressure (Ahlquist *et al.*, 1994). The pressure was regulated through 2 valves to ensure consistent application and the housing for the dentine disc has also been modified to reduce leakage. The system could be recommended as suitable for analysis of toothpaste or mouthwash which anticipated dentine tubule occlusion.

The control samples in this experiment received no treatment after establishing the baseline hydraulic conductance of the samples. It is interesting to note however that in the work of Santiago et al (2006), investigating various concentrations of potassium oxalate gel, the control samples were treated with water. The hydraulic conductance rates were then re-tested after 5 minutes by which time the hydraulic conductance had reduced by 12.04%. After 15 minutes the hydraulic conductance had reduced by a further 13.93% and at 30 minutes by a further 10.34%. It therefore perhaps would have been advisable in the present study to allow a period of time after baseline flow rate measurement before re-testing the control samples, to record any natural fluctuation in hydraulic conductance that could not be attributed to the in situ housing. The control samples were not brushed with water to avoid unnecessary damage that may form on the surface by brushing as is suggested to have been the case in the work carried out by Wang et al (2010).

The use of SEM imaging for the assessment of tubule occlusion is commonplace for both in vitro and in situ studies seeking to quantify the treatment potential of toothpaste agents in tackling DH. The mode of action of the agent can dictate the suitability of SEM imaging as a logical choice for testing. Section 1.5 outlines the most common means of occluding dentine tubules, often this is through the creation of a barrier at the dentine surface as a means to limit the hydrodynamic flow of dentinal fluid and subsequent transmission of stimuli to the pulpal nerves. It has previously been shown through an in vitro study by Arnold et al (2015) that the agents strontium acetate, stannous fluoride, and zinc-carbonate hydroxyapatite were able to occlude dentinal tubules. This study employed SEM imaging from a top down perspective as well as a cross sectional view to observe penetration of the active ingredients delivered by toothpaste into the dentine tubules. The results showed that strontium acetate produced occluding plugs in most of the dentine tubule openings which extended up to 15 µm into the tubules. Stannous fluoride demonstrated no occlusion when viewed as a cross section and only small numbers of individually occluded tubules from top down perspective. Regarding zinc-carbonate hydroxyapatite, it was found that multiple occluded dentine tubules could be observed both in the longitudinal and surface SEM images extending up to 5 µm into the tubules. The investigation suggests that all agents tested were able to occlude the tubules but none offered a complete coverage. The present study found evidence of crystalline structure deposits in multiple dentine tubules from samples treated with potassium oxalate. The depths of the deposits ranged from 5 µm to a maximum of 25 µm into the dentine tubules however crystals were not observed at the dentine surface.

Dentine samples used for in situ occlusion studies where SEM is used to determine the level of occlusion are often manufactured from root dentine in contrast to the coronal dentine discs necessary for the present study (Olley *et al.*, 2012; Seong *et al.*, 2013). In these studies prior to the in-situ phase, the dentine surface is examined under SEM to establish tubule patency and check orientation of tubule openings, if the tubule outlines are not judged to be circular or at a 90° angle to field of view the sample is discarded (Banfield and Addy, 2004). Samples that are taken through to the in-situ phase are therefore ready to be treated with the study regimen.

The occlusion studies as reported by Olley et al (2012) and Seong et al (2013) applied the treatment toothpaste to the dentine samples ex vivo followed by an acid challenge. Following the treatment phase and exposure to the oral environment, individual dentine samples were removed from the appliance for evaluation. SEM images were taken from a central location on the dentine surface. These images were scored for levels of occlusion by examiners blinded to the sample treatments. The scoring system employed was as follows: 1 = occluded, 2 = partially un-occluded, 3 = equally occluded/unoccluded, 4 = partially occluded and 5 = unoccluded. The 5 point scoring system therefore allows for an overall impression of the levels of occlusion to be scored without examining each individual tubule in detail. Here lies a divergence with the present study, which used a binary 2 point scale for occlusion scores. Some of the samples scored as unoccluded contained 1 or more tubules which were occluded even though the majority were not. The more descriptive 5 point scale may have been preferable to give an option to score partial occlusion.

The SEM images taken in this present study were taken from a longitudinal view of a fractured dentine disc and the number of individual visible tubules was <20. In the work of Seong and Olley the numbers of tubule openings visible from the top down viewpoint varies and depends on the level of magnification but is often >100. The numbers of tubules examined were significantly lower in the present study because most of the imaging area was occupied by intertubular and peritubular dentine extending up to 80 µm into the tubule, this was as a result of the disc shaped specimen and fracturing technique exposing a limited area.

The initial interrogation of the surface of the dentine discs for this study was performed using FIB-SEM combined with EDX analysis. This technique was conducted to examine the infiltration of deposits into the dentine tubules as well as the depth to which these deposits were present. Where deposits were found EDX analysis was performed to map the chemical composition of specific sites. The FIB can be used to mill away sections of material in areas defined by the user to expose a cross section for analysis. A previous study performed in vitro was successful in employing this technique to confirm deposits of strontium incorporated into the dentine tubules to a depth of at least 15 µm, following treatment with a toothpaste containing 8% strontium acetate and 1040 ppm sodium fluoride (Earl *et al.*, 2010). Visual interpretation of the FIB-SEM data from the present study suggested possible infiltration of the potassium oxalate into the dentine tubule and consequent deposits of calcium oxalate crystals as seen in figure 3.4 B and 3.6 A and B. Previous in vitro research has shown that potassium oxalate occludes dentinal tubules by creating acid-resistant calcium oxalate crystals (Pashley and Galloway, 1985a; Mongiorgi *et al.*, 1992; Gillam *et al.*, 2001; Sauro *et al.*, 2006). The crystal structures were examined with EDX to determine their chemical composition. The mass percentage of hydroxyapatite is as follows: calcium 39.89%, oxygen 41.41%, hydrogen 0.20% and phosphorus 18.50% (WebQC, 2018). Human dentine consists of 70% by weight calcium-deficient carbonate apatite crystals, $Ca_{10}(PO_4)_6(OH)_2$ (Pashley, 1996). If the crystal structures found were calcium oxalate crystals a significant increase in calcium levels would be expected. It should also be noted that the spot size of the site being investigated and depth of penetration when using an EDX instrument may mean that elements from the surrounding area as well as the participate itself can be collected making the data less accurate. Never the less, the EDX technique has found success in dentine samples treated with arginine in combination with calcium carbonate. In one study the openings to dentine tubules were found to be occluded when viewed from the top down under SEM and chemical mapping demonstrated that the surface layer on the dentine consisted of additional calcium and phosphate and had penetrated to a depth of 2 µm (Petrou *et al.*, 2009).

In the present study there were several sites which reported increased calcium levels across multiple dentine tubules. The highest percentages of calcium were 79.33%, 65.84% and 64.95% found at sites 1, 2 and 3 respectively from figure 3.4 B and recorded in table 3.1. The site number 3 from a different sample shown in figure 3.6 B reported 61.78% calcium in table 3.3. These figures report calcium deposition between 20-40% higher than the 39.89% found naturally in hydroxyapatite. As these calcium spikes were accompanied by visual evidence of crystalline formations it can by hypothesised that the potassium oxalate treatment successfully penetrated the dentine tubules allowing for ion exchange to occur between potassium and calcium leading to the formation of calcium oxalate crystals.

Although, it should be noted that many of the sites measured on both treated and untreated samples recorded reported mass percentages at very similar levels to those of hydroxyapatite, with levels of calcium around 40%. It can be theorised that the FIB-SEM milling process redeposited the milled hydroxyapatite back onto the exposed tubule walls. This hypothesis is supported by the findings of Earl et al (2010) who reported that FIB milling is a destructive process and sputtered material will collect on the surfaces of tubules. The number of tubules investigated was low due to the time and cost associated with the technique and the chemical profile failed to show a significant number of deviations from that of hydroxyapatite. Nevertheless, the spikes in calcium levels found in 2 of the 3 treated samples point towards the possible usefulness of developing the technique for use in future studies which seek to quantify treatments applied to dentine.

Following the FIB-SEM and EDX investigation all 33 samples were prepared for examination under conventional SEM through the process of fracturing and mounting each sample. This technique has been successful for the imaging of calcium oxalate crystals found during in vitro studies. Arrais et al (2004) reported substantial crystal-like deposits within the dentine tubule lumen extending to a depth of 15 µm after treatment with a potassium oxalate gel. The crystal dimensions varied in diameter and some occluded across most of the tubule diameter. A further study also employed a fracturing technique involving air drying dentine discs and bending them between two pairs of forceps to expose the dentine tubules (Hare *et al.*, 2016b). Following treatment with potassium oxalate gel this study found well-formed crystalline structures between a depth of 5 µm to 25 µm from the dentine surface and it was noted that multiple applications of oxalate gel resulted in higher crystal density which appeared to occupy the entire dentine tubule lumen. Both studies by Arrais and Hare reported that untreated control samples displayed no signs of tubule occlusion at the surface or inside the tubules.

The SEM images of the fractured dentine discs in the present study can be seen to corroborate these findings in regard to the samples treated with potassium oxalate gel. The 3 examiners blinded to the sample treatments were unanimous in their scoring of the treated images, giving each image a score of 1 or occluded, as stated in table 3.5.

The images of treated samples presented above in figure 3.9 contain crystal formations, highlighted with red arrows, which appear to form complete blockages across the entire diameter of multiple tubules at depths ranging between 2 µm and 25 µm, the crystals being most evident in figure 3.9 A, C and D. In these images the crystals are large enough to fill the tubule lumen with a single crystal. In figure 3.9 B and E a number of smaller crystals combine to span the tubule diameter at depths between 5 µm and 35 µm These formations appear to mirror the crystalline structures found in the work of Arrais et al (2004), Hare et al (2016b) and Pereira et al (2005). It should also be noted that crystal structures were not apparent in every sample treated with potassium oxalate in this study as it was only a small cross section of the sample that was available for viewing, however the images exhibited in figure 3.9 are representative of the formations found in treated samples. The images presented here support the findings of the flow cell segment of this study where a statistically significant reduction in hydraulic conductance was found following the in situ phase of the study for both treated and non-treated samples.

The results of the present study include SEM images of each dentine sample which had been mounted into the appliances during the in situ phase. A visible layer of debris was present in some of the images found in section 3.4.4 which is likely to have formed as a result of the participant's dietary intake whilst wearing the appliance. It is hypothesised that this debris layer largely contributed to the reduction in hydraulic conductance noted for both treated and un-treated dentine discs following removal from the palatal appliance.

In most of the currently reported in situ occlusion studies, the oral appliance is worn for a maximum of 8 hours per day and removed for eating and drinking anything other than water (Seong *et al.*, 2013). These are two of the fundamental differences between some of the other in-situ studies and the present one. In this investigation the appliances were worn continuously including during the hours of sleep, apart from when the participants brushed their own teeth, cleaned their appliance or where the appliance was cleaned by study site staff. The only restrictions on diet were to avoid very sticky or chewy foods such as toffee or chewing gum and there were no restrictions on beverage consumption. In hindsight this study would have benefitted from requesting the participants to keep a diary of their diet and perhaps additional restrictions on dietary choice. This would have provided further information relating to the nature of the debris found on the dentine samples as described in section 3.4.4. However, the study was seeking to reproduce the in vivo effect of diet and the oral cavity on the dentine samples as closely as possible. A previous in vitro study performed on bovine enamel to establish the effect of daily diet on tissue loss found that different combinations of food and drink have different effects on the degree of enamel loss (Forbes-Haley *et al.*, 2016). To our knowledge there has been no data of in situ studies investigating dentinal tubules occlusion where the appliance was worn whilst eating.

This study was conducted over an extended duration for the in situ stage (14 consecutive days) compared to the other occlusion studies that have been conducted over 4 days (Olley *et al.*, 2012; Seong *et al.*, 2013). A 4 day cycle would require less participant compliance however it would not have established the resilience of the potassium oxalate treatment used in the present study as the dietary intake would be considerably more limited over this shorter time period.

The continuous 14 day duration allowed the dentine samples to become immersed in a pellicle layer and to withstand the full daily cycle of acidic challenges and rest periods. This tested the ability of the potassium oxalate treatment to provide durable occlusion resistant to challenges in the oral environment. Moreover, previous in situ models investigating the anti-erosive effect of different toothpastes have successfully adopted the 15 day duration (Hooper *et al.*, 2014; Bellamy *et al.*, 2014) therefore it can be seen to strike a balance between participant comfort and sample exposure to the oral environment.

The design of the palatal appliance used in this study purposefully left the coronal side of the dentine discs open to the oral cavity by protecting the sample with 4 parallel wires allowing saliva and dietary components the ability to interact with the sample, as would be the case in vivo. As previously stated the appliance was cleaned daily by both the participant and study site staff. In conversation with the participants and as evident by the condition of the appliance before cleaning the diet of the participant was a significant factor in the status of the appliance throughout the in situ phase. In particular the upper ridge of the palatal aspect trapped food debris although this was dependant on the shape of the participant's oral cavity. Also, the slot used for loading of the dentine discs into the appliance was only partially filled with wax to limit the risk of the wax disintegrating but this allowed space for the collection of food detritus. Several participants reported that the clasp on the molar tooth holding the appliance in place could be bitten down on during mastication causing the body of the appliance to rise up and allow food particulate to become trapped between the appliance and roof of the mouth. This issue was dealt with by the study clinician by adjusting the clasps however with some participants the problem persisted. The appliance was cleaned using a manual toothbrush and water which successfully removed any food deposits, although great care was taken to avoid brushing the dentine disc as much as possible to prevent damage to the delicate dentine surface. Over the duration of the study however some of the dentine samples changed from their original colour as described in section 3.4.6. It is therefore highly likely that food debris was able to penetrate some of the dentine discs which may explain to an extent the reduction in fluid flow from samples which received no treatment. This was also confirmed by the SEM images indicating a debris layer on the dentine surface of both treated and non-treated samples. Any future work would need to involve a redesign of the appliance to better protect the samples from dietary constituents while balancing the need for the dentine to be exposed to the oral environment to replicate in-vivo conditions.

The participant's entry into the in situ phase was staggered over 2 weeks to allow sufficient time to measure the baseline flow rate of each sample, treat and mount the dentine discs into the oral appliance. Within 24 hours of the first participant starting it became apparent through conversation with the participant and inspection of their appliance after eating that it would be necessary to include an antiseptic stage in the daily cleaning regime by the study site staff, this extra stage was introduced for all subsequent participants. The specific time of appliance cleaning was by arrangement with the individual participant but was generally after they had eaten lunch. Following the cleaning with a toothbrush and tap water the appliance was immersed into a chlorhexidine solution containing 0.2% chlorhexidine digluconate (Corsodyl, GSK, Brentford, UK) for 2 minutes then rinsed with water before returning to the participant. The technique was based on one used previously for an in situ study (Hooper *et al.*, 2014). This was intended to prevent the growth of microorganisms and leave the appliance with a mint taste.

The study of dentine hypersensitivity in vivo has opened numerous avenues of investigation. It has been suggested that if the hydrodynamic theory is correct and dentine is therefore permeable across the entire length of the tubules, then the diffusion of bacterial products through the dentine to the pulp and any subsequent contamination is possible and will elicit pulpal reactions (Bergenholtz, 1981; Pashley, 1992b; Vojinovic *et al.*, 1973). The slow outward movement of dentinal fluid will act to flush the tubules free of bacteria, although an inflammatory response by the pulp will increase this fluid flow which could be distinguished as pain (Maita *et al.*, 1991). In the present study however the dentine discs placed in situ were slotted up against the acrylic appliance at the pulpal side, with no available reservoir of fluid and therefore no outward flow would have been present to flush any bacteria or debris. When considering the reduction of fluid flow found in untreated samples and the appearance of a smear layer and sample discolouration that was identified by SEM and by the human eye it can be postulated that despite the cleaning regime that took place, bacterial growth occurred which in turn reduced the fluid flow through the dentine discs. This hypothesis is supported by in vitro studies investigating the penetration of dentine tubules by bacteria. Michelich (1980) was successful in demonstrating that where bacteria invaded dentine tubules the fluid flow through the tubules was reduced. Furthermore, in a study of the penetration of streptococcus gordonii into dentine sections over 21 days the hydraulic conductance of fluid through the dentine was reduced by 42% (Love *et al.*, 1996). The focus of this study was both assessing the efficacy of the treatment product and the suitability of the flow cell model for investigation of occluding agents. Consequently, there was no scope in the present investigation to analyse bacterial penetration into the dentine tubules, however this would be of considerable benefit in future work.

4 Summary Discussion

The primary objective of this study was to determine the efficacy of a gel strip treatment impregnated with 3.14% potassium oxalate in occluding dentine discs. The mode of action for this treatment was the occlusion of the dentine tubules and the hydraulic conductance of dentine disc samples was quantified for evidence of a reduction in flow between baseline and post-treatment measurements. Further to this, the study aimed to investigate the capability of the flow cell model to provide quantifiable data relating to the effectiveness of occlusion based treatment applied to a dentine discs, whilst determining the ability of the system to differentiate between treated and non-treated discs following a 14 day in situ phase. The findings from the flow cell phase were corroborated through the use of longitudinal FIB-SEM micrographs and EDX chemical mapping of sites within the dentine discs. This investigation was further expanded to include SEM micrographs of fractured dentine discs, subsequently scored for levels of occlusion by blinded examiners.

Prior to undertaking the in-situ experiment a pair of investigations into sample preparation and surface brushing techniques were undertaken to provide a robust protocol for future work using the flow cell model. These investigations determined the acid solution used for etching of all future dentine disc samples and provided a standardised technique for sample surface brushing.

The sectioning of whole human teeth to produce dentine samples leaves behind a smear layer composed of debris and proteins on the dentine surface (Pashley, 1992a). This smear layer must be removed to leave dentine tubules patent and allow for the uninhibited flow of fluid through the dentine. In the present study 3 solutions were tested for their ability to remove the smear layer and open the dentine tubules. These were acetate buffer solution, 10% citric acid solution and 6% citric acid solution. Samples etched with the 10% citric acid solution for 30 seconds produced consistent flow rates through the dentine which were substantially reduced following treatment with the potassium oxalate gel. However when viewed under SEM the dentine surface appeared rough and had possibly demineralised, a finding supported by the work of Reis et al (2008). The samples that were submerged into a 6% citric acid solution for 4 minutes under ultra-sonication also produced flow rates consistent with each other which were considerably reduced following oxalate treatment. 6% citric acid is commonly used to remove smear layer in studies examining the occluding potential of different agents (West *et al.*, 2011b; Olley *et al.*, 2012; Sharma *et al.*, 2013a) and proved a suitable choice for further work in this investigation. The samples etched with acetate buffer solution for 120 minutes reported flow rates far below the other solutions tested. A further reduction in flow rate after treatment was noted, however the difference from the baseline readings was not to the same extent as both other groups. Acetate buffer solution has been shown to successfully remove the smear layer from dentine in the work of Shellis and Curtis (2010). Further work would be required to understand the level of smear layer removal that was taking place using this solution therefore for this study it was decided to use the 6% citric acid solution for all future work.

Once the dentine disc samples had been mounted into the flow cell it was necessary to perform a series of surface brushing procedures prior to measuring the baseline hydrodynamic flow rate. This involved the creation of a pellicle layer followed by a series of brushing regimes using an electric and manual toothbrush to stabilise drift in hydraulic conductance and remove any deposits of crystallisation from the HS, a technique supported by the work of Hare et al (2016b). This approach cannot be found in other similar in vitro work (Santiago *et al.*, 2006; Pereira *et al.*, 2005; Varoni *et al.*, 2017), possibly because these studies used DI water for filtration through the dentine samples which would not contain agents likely to deposit on the surface and was therefore not required.

It was noted in preliminary work that the first of three baseline flow rate measurements taken from the same sample often differed from successive measurements. It was hypothesised that this was because the surface brushing protocol that was being followed prior to the first baseline measurement was longer than the brushing protocol followed in between each baseline measurement. It was therefore decided that performing a full surface brushing cycle between each set of measurement would be tested. The application of this revised protocol led to a deterioration in the silicone washer used to seal the dentine disc into the cell as well as an over saturation of the sample with HS. Also, the first flow rate measurement continued to differ from the successive measurements, therefore the original brushing protocol was maintained throughout subsequent experiments. Consequently, a fourth flow rate measurement cycle was added to all the following research in the present study to provide an extra data point in the calculations of mean flow rates to better allow for variation in measurements.

Investigations into the mechanism of action for agents that have the potential to reduce DH through occlusion of dentine tubules require a standardised sample preparation and processing procedure. In the present study a standardised procedure has been developed, which produces dentine samples with a polished surface for subsequent flow rate investigation and SEM imaging. Samples were prepared using a standardised methodology to reduce anatomical variation. The flat samples used in this study were beneficial for imaging dentine tubule occlusion. Imaging acquisition was also standardised using longitudinal SEM and FIB-SEM images taken from different sites on each sample.

The examination of the potassium oxalate treated dentine discs using FIB-SEM allowed for a limited number of tubules to be scrutinised in detail following treatment with potassium oxalate. Visual analysis suggested possible formation of crystal like structures within the tubules and in some of these locations chemical spectroscopy reported increased levels of calcium of between 20-40%, perhaps suggesting ion exchange the deposition of calcium oxalate crystals at those sites.

However, in some treated samples the chemical analysis failed to differentiate between these precipitates and hydroxyapatite. A similar chemical composition was found in both treated and un-treated dentine discs for some of the sites tested. The technique has proved to be successful when examining dentine samples treated with a toothpaste containing 8% strontium acetate and 1040 ppm sodium fluoride (Earl et al., 2010). It could therefore warrant further testing across greater sample numbers following treatment with occluding agents such potassium oxalate.

Treatment modalities designed to desensitise the tooth have one or two modes of action: nerve sensitisation and tubule occlusion (Krauser, 1986). Many treatment modalities have focused on agents which act to desensitise the dental nerves such as various potassium salts (Schiff *et al.*, 1994). However, the evidence for the use of potassium salts in the management of DH is unclear as it is not easy to investigate the uptake of desensitising agents and it is complicated by the outward flow of dentinal fluid (Orchardson and Gillam, 2000). Moreover, it has been shown that in individuals suffering from DH, the tubules are larger, more penetrable and more densely packed resulting in further development of products aiming to occlude dentinal tubules and thus treat DH (Absi *et al.*, 1987a). As a result, many in vitro and in situ studies have been undertaken to determine the efficacy of occluding agents. Several methods can be used to visualise the dentine tubule occlusion with SEM imaging and scoring of the images being the most popular analysis (Olley *et al.*, 2012; Seong *et al.*, 2013; Chen *et al.*, 2015).

In the present study the hydraulic conductance data following the in situ phase reported a reduction in fluid flow through samples which received the potassium oxalate treatment as well as those which received no treatment. The reduction in fluid flow through samples that had not been treated could conceivably be attributed to the fact that saliva naturally transports calcium and phosphate ions into dentinal tubules. This may result in the formation of a surface protective layer or pellicle comprised of salivary glycoprotein containing calcium and phosphate which in turn may lead to the natural occlusion of patent dentinal tubules (Cummins, 2010). Nevertheless, this process of natural tubule occlusion is very slow and the efficacy of the tubule occlusion is questionable as it is easily removed by dietary and physical challenge. It can be hypothesised that the reduction in permeability noted in section 3.4.2 following the in situ phase was in part due to debris from food and drink adhering to the dentine surface. This provides important information regarding the appliance design used for in situ studies where the appliance is not removed for eating and drinking.

Oxalates were presented as agents to treat DH in the late 1970s based on in vitro research (Cunha-Cruz *et al.*, 2011). Research has suggested that oxalates limit fluid flow in exposed dentine, thereby reducing pain by significantly decreasing hydraulic conductance of fluid through dentine treated with oxalates (Pashley *et al.*, 1978; Greenhill and Pashley, 1981; Pashley and Galloway, 1985a). Oxalates have demonstrated the ability to form precipitates within dentine tubules which, as a result, impede dentinal fluid flow (Cuenin *et al.*, 1991; Santiago *et al.*, 2006). These deposits were advantageously characterised by relative insolubility in acid, making them resistant to dissolution after treatment (Pereira *et al.*, 2005). Hare et al. (2016b) found that potassium oxalate caused a reduction in hydraulic conductance in vitro and formed deposits with strong occluding performance. This finding was dependant on the oxalate application time and frequency of application. The results were tested over a 30-day in vitro cycle, showing an excellent resistance to dissolution and mechanical challenges that the dentine was subjected to throughout this period. In the present study the samples treated with potassium oxalate retained and increased the reduction in fluid flow rate from baseline and this can therefore be seen to support the work of Pereira et al (2005) and Hare et al (2016b).

RCTs have tested the use of oxalates in self-administered mouth rinse (Sharma *et al.*, 2013b), self-administered strips (Papas *et al.*, 2016) and in professionally applied treatment in short term RCTs (Craig *et al.*, 2012) and long term RCTs (Merika *et al.*, 2006; Gillam *et al.*, 2004; Pamir *et al.*, 2007; Vieira *et al.*, 2009; Erdemir *et al.*, 2010; Camilotti *et al.*, 2012; Vora *et al.*, 2012). The results from these studies have shown an efficacy in reduction of pain sensation by alleviating DH for the participants involved. The data from the present study indicating a significant reduction in fluid flow following treatment with oxalates highlights the possibility of taking the gel strip application technique into further research conducted as RCTs to attain data comparable with the above in vivo studies.

The potassium oxalate treatment in this study was under investigation for its ability to occlude dentine tubules. It is essential to note that a second neural effect has been suggested by several authors (Peacock and Orchardson, 1999; Pashley, 1986; Pereira and Nicolau, 1993; Amini *et al.*, 2016). The results of treatment with potassium oxalate is neural depolarisation from potassium ions and the later formation of calcium oxalate crystals. Neural depolarisation takes 2 weeks to show any effect clinically (Muzzin and Johnson, 1989). It is not possible to measure this effect using the model applied in this study and the reduction in dentine permeability found after treatment with potassium oxalate in the present study is not related to this finding. However, it is important information for the treatment of DH in patients and points towards possible further avenues of in-vivo testing using potassium oxalate strips in RCTs where pain response of participants could be investigated.

The use of adhesive strips has been reported as one option for application of fluorides to treat dentine hypersensitivity (Lee *et al.*, 2013) as well as a whitening agent for home bleaching purposes (Donly, 2010). In the present study the strip was used because it enabled localised application of the treatment agent onto the dentine surface. For in vivo application this would allow the treatment desensitising agent to target specific teeth. It can provide the convenience of self-administered application or professional application at sensitive sites. In addition, these strips provide a means to hold the agent in place to allow sufficient penetration of potassium oxalate into the dentinal tubules and do not involve brushing the surface which may lead to hard tissue surface loss (Azevedo *et al.*, 2008; Choi *et al.*, 2012).

An effective and durable occlusion result was discovered when potassium oxalate was applied as a strip for dentinal tubule occlusion in comparison to control group which received no treatment. This is in agreement with in vitro studies by Hare et al (2016b) and the in vivo study by Papas et al (2016). The study results can be attributed to precipitation of calcium oxalate crystals forming that are able to adhere to the dentine surface and occlude the dentinal tubules (Varoni *et al.*, 2017) and suggests the flow cell model could be proposed for the testing of other dental products such as toothpaste or mouthwash which contain occluding agents. The SEM images from the present study corroborate the flow cell data as evidence of crystalline formations was only found in those samples treated with potassium oxalate.

5 Conclusion and Future Work

5.1 Conclusion

The results from this study provide compelling in vitro evidence for the effective occlusion of dentine samples by the application of a gel strip containing 3.14% potassium oxalate, significantly reducing the hydraulic conductance of fluid flow through the dentine tubules. A further reduction in fluid flow was found after a 14 day in situ phase where the dentine samples were continuously exposed to the oral environment whilst being housed in a palatal appliance. This finding was corroborated by SEM imaging which found evidence of crystalline structures penetrating up to 35 µm into the dentine tubules with multiple crystals filling the width of the tubule lumen. The micrographs taken with FIB-SEM and analysed with EDX depicted some evidence of dentine tubule occlusion and the chemical spectroscopy suggested increases in calcium levels at some sites but failed to do so at others. The technique would require further calibration with dentine samples to provide accurate data.

Samples which received no treatment also significantly reduced dentine permeability following the in situ phase but not to the same degree as the treated samples. The SEM images showing the cross sectional view of the untreated dentine did not show the same crystal formation within the tubules. The palatal appliance design whilst functioning well for a standardised day, did not fare so well for continuous use due to food accumulation in the sample loading slot, suggesting that the oral hygiene regimen for the appliance could be improved. This finding was supported by discovering a debris layer on both treated and un-treated samples when viewed under SEM and by eye. The difference in hydraulic conductance between treated and untreated samples following the in situ phase showed that the degree of occlusion was significantly greater for the treated samples thus providing evidence that the potassium oxalate strip was effective in occluding tubules over an extended period of time. The flow cell model was able to show the durability of the potassium oxalate treatment whilst also differentiating between the fluid flow of treated and non-treated dentine surfaces.

5.2 Future Work

Results from these studies highlight areas of future research, suggesting new avenues for investigation.

These studies were conceived as a proof of concept utilising a flow cell to measure the occlusion potential of potassium oxalate in the reduction of fluid flow through dentine in situ and therefore the inferred reduction of pain in vivo. The flow cell apparatus would be suitable for investigation of other DH treatments seeking to limit fluid flow via the occlusion of dentine tubules such as a toothpaste or mouthwash. A comparison of other potential occluding agents could be carried out utilising the in situ model.

The palatal appliance used for the in situ period of this study was novel and designed to protect the dentine discs from breakage whilst leaving the discs exposed to the oral environment. As has been discussed the hydraulic conductance through the discs was probably affected by a debris layer comprised of the dietary intake of the participants. Future studies seeking to utilise an oral appliance in a study where the participants keep the appliance in their mouth whilst eating and drinking would need to modify the disc housing to balance protection from dietary intake with exposure of the discs to the oral environment. Any changes to the appliance design should be considered alongside the disinfection and cleaning regime of the appliance, without disrupting the oxalate deposition on the disc. A diary of the participant's diet throughout the in situ phase could also help to build a clearer picture of the effects of the oral environment on each dentine sample. It would also prove invaluable to include investigation of any bacterial penetration into the dentine samples following removal from the oral appliance to provide an understanding of any effect this may have.

In the present study crystalline formations were observed in dentine tubules from the fractured surfaces of dentine samples, which mirrored those found in other surface studies. EDX chemical mapping of these crystals to confirm the presence of calcium oxalate on the cross sectional SEM images would be highly desirable in future studies and would provide confirmation that the deposits within the tubules are calcium oxalate crystals.

The results from this study suggest that potassium oxalate reduces fluid flow through dentine discs when measured in vitro. To establish the treatments suitability for reduction of pain caused by DH it could be suggested to undertake a clinical trial using gel strips containing potassium oxalate in vivo. For the purposes of ensuring a robust investigation the study would need to include a number of informed and consented participants large enough to produced statistically significant data. The study design would need to randomised and double blind with the inclusion of a control group.

5.3 Conflict of Interest and Funding Statement

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Appendix I: Participant Brushing Instructions

Study 2015075: A Clinical Study to Evaluate the Efficacy of an Oxalate Strip on Dentinal Hypersensitivity product instructions
Version 2.0, 29th March 2016

<u>Take Home Product – Usage Instructions</u>

Today you have been given a KIT BOX containing a Toothbrush and Toothpaste.

- Beginning today, please use the products you have been provided with in place of your regular toothbrush and toothpaste.
- Brush your teeth twice a day (morning and evening), using <u>only</u> the products provided.
- Please use these products for the duration of the study.
- If you are running out of toothpaste or require a replacement toothbrush, please notify the study staff.
- At the end of the study at follow-up, please return the Kit Box with all used/unused product (toothbrush and toothpaste) to the study site.

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Brushing Instructions:

- Brush your teeth, as you normally do, twice a day;
- After brushing rinse your mouth with water to remove excess paste.

**** Please abstain from the following during the study duration:***

- Non-study oral care products (including toothbrushes and toothpastes, mouth rinses, floss, and toothpicks for plaque removal;
- Do not participate in any other oral/dental products study
- Delay any elective dentistry (including dental cleanings)

- **Please, RETURN** your kit box and the contents to the study site at your **FOLLOW-UP** visit;

[redacted]

Thank you for your Participation in this Clinical Study

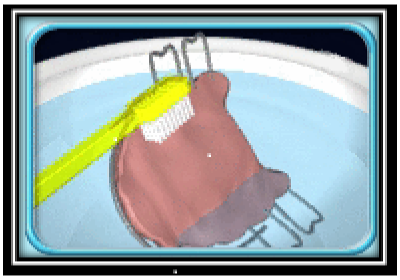
Appendix II: Palatal Appliance Care Instructions

Study 2015075: A Clinical Study to Evaluate the Efficacy of an Oxalate Strip on Dentinal Hypersensitivity
Appliance Care Instructions
Version 2.0, 29th March 2016

STUDY 2015075

PALATAL APPLIANCE CARE INSTRUCTION SHEET

Figure 1:



Caring for your palatal appliance:

- The palatal appliance (removable brace) should be worn at all times of the day and night, including mealtimes and whilst sleeping, unless otherwise instructed.
- Please remove your brace while you clean your teeth, then replace in your mouth once teeth cleaning is complete.
- You will need to clean your brace at least twice a day. Rinse the appliance under the tap after every meal where possible and clean it with your provided toothbrush.
- NB Only use water and the toothbrush provided to clean your brace (see Figure 1).
- Sticky and very chewy foods can damage the appliance and dentine samples, eg. toffee and chewing gum. Please avoid such foods.
- If the brace or dentine sections are damaged, please contact the study site as soon as you can.

Appendix III Flow Rate Data (µl/min)

	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	Sample 13	Sample 15	Sample 17	Sample 19	Sample 21	Sample 23	Sample 25	Sample 27	Sample 29	Sample 31	Sample 33	Sample 35	Sample 37	Sample 39	mean values	mean values with exclusions
mean baseline	85.94	49.53	198.07	41.53	292.57	100.50	18.40	87.53	39.93	39.71	134.97	91.16	68.01	65.48	80.13	47.35	23.59	186.96	17.29	48.32	85.8	90.3
stdev	2.95	2.72	3.72	0.22	33.08	1.19	0.17	0.76	0.40	0.82	1.63	1.48	0.90	0.42	2.31	0.89	1.47	11.64	1.68	9.86		74.1
mean post tx	51.37	12.08	50.87	18.40	135.90	38.16	6.27	25.83	14.26	17.66	61.22	59.01	25.96	40.26	31.83	29.30	3.39	46.02	1.20	26.21	34.8	36.7
stdev	0.62	0.03	0.59	0.08	2.13	0.15	0.03	0.26	0.04	0.15	0.56	0.98	0.15	0.23	0.81	0.17	0.50	2.87	0.17	1.73		31.8
mean post appl	21.96	4.63	0.67	23.15	4.36	225.05	0.16	13.77	18.44	2.99	23.21	25.10	26.09	1.58	1.56	26.19	0.16		0.83	0.36	22.1	9.6
stdev	0.75	1.14	0.06	1.37	1.48	27.60	0.00	1.18	0.37	0.22	1.42	0.78	2.59	0.08	0.06	1.12	0.01		0.02	0.01		10.9
BL - PT	34.57	37.45	147.20	23.14	156.67	62.34	12.13	61.70	25.67	22.05	73.76	32.15	42.05	25.22	48.31	18.05	20.20	140.94	16.09	22.11		48.1
BL - PA	63.98	44.90	197.40	18.39	288.21	-124.55	18.24	73.76	21.49	36.72	111.76	66.06	41.92	63.90	78.57	21.16	23.43	186.96	16.46	47.96		74.7

	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	Sample 22	Sample 24	Sample 26	Sample 28	Sample 30	Sample 32	Sample 34	Sample 36	Sample 38	Sample 40	mean values	mean values with exclusions
mean baseline	110.06	37.10	116.56	76.42	34.66	222.44	300.16	176.61	117.55	51.58	96.36	52.62	39.37	4.14	43.35	327.26	56.76	183.27	235.35	23.50	115.3	123.8
stdev	1.50	0.44	0.95	1.22	0.29	13.97	32.17	43.11	25.94	1.02	1.68	0.54	0.31	0.63	4.01	19.97	2.53	10.47	14.73	1.55		96.4
mean post appl	38.57	62.42	1.43	7.73	16.21	21.58	243.55	181.33	15.95	4.13	23.65	40.93	195.42		2.76	73.35	2.22	2.03	2.38	2.63	49.4	40.0
stdev	1.85	7.95	0.11	0.30	1.61	1.25	57.76	4.50	0.84	1.35	3.47	2.23	33.11		0.03	11.05	0.36	0.08	0.70	0.06		68.5
BL - PA	71.50	-25.32	115.14	68.68	18.44	200.85	56.61	-4.71	101.60	47.46	72.71	11.69	-156.05	4.14	40.59	253.91	54.55	181.24	232.97	20.88		90.8

	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	Sample 22	Sample 24	Sample 26	Sample 28	Sample 30	Sample 32	Sample 34	Sample 36	Sample 38	Sample 40	mean values	mean values with exclusions
Baseline 1	111.9	37.8	117.6	74.7	34.3	234.1	334.3	128.0	156.2	50.2	96.7	52.8	39.5	4.5	47.8	338.7	59.8	191.3	235.2	25.4	118.5	127.4
Baseline 2	109.6	36.9	116.2	77.4	34.8	231.0	256.6	173.9	107.6	51.4	96.8	52.6	39.7	4.7	45.5	348.2	57.5	189.9	256.1	24.0	115.5	124.1
Baseline 3	110.4	36.9	115.5	76.3	34.6	203.1	305.9	233.0	106.2	52.1	98.0	53.2	39.3	4.0	41.1	303.9	56.0	183.4	227.2	22.9	115.1	123.7
Baseline 4	108.3	36.9	117.1	77.3	34.9	221.5	303.8	171.5	100.3	52.6	94.0	51.9	39.0	3.3	39.0	318.3	53.8	168.4	222.9	21.8	111.8	120.0
Post Treatment 1	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
Post Treatment 2	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
Post Treatment 3	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
Post Treatment 4	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
Post Appliance 1	38.5	72.5	1.5	8.1	17.1	23.2	327.5	178.6	16.9	5.7	28.2	40.6	244.6	#VALUE!	2.8	89.1	1.9	2.0	1.5	2.7	58.0	46.2
Post Appliance 2	38.9	63.9	1.4	7.8	17.3	22.0	211.1	185.6	16.4	4.7	24.5	41.6	179.9	#VALUE!	2.7	68.1	2.1	2.0	2.1	2.5	47.1	38.3
Post Appliance 3	40.7	59.9	1.3	7.3	16.7	20.8	200.6	184.7	15.1	3.4	21.2	43.4	184.0	#VALUE!	2.8	72.4	2.2	2.1	2.8	2.7	46.5	37.7
Post Appliance 4	36.2	53.4	1.5	7.7	13.8	20.4	235.0	176.5	15.4	2.7	20.7	38.1	173.2	#VALUE!	2.8	63.8	2.7	2.1	3.1	2.6	45.9	38.0
	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	Sample 13	Sample 15	Sample 17	Sample 19	Sample 21	Sample 23	Sample 25	Sample 27	Sample 29	Sample 31	Sample 33	Sample 35	Sample 37	Sample 39	mean values	mean values with exclusions
mean baseline	85.94	49.53	198.07	41.53	292.57	100.50	18.40	87.53	39.93	39.71	134.97	91.16	68.01	65.48	80.13	47.35	23.59	186.96	17.29	48.32	85.8	90.3
stdev	2.95	2.72	3.72	0.22	33.08	1.19	0.17	0.76	0.40	0.82	1.63	1.48	0.90	0.42	2.31	0.89	1.47	11.64	1.68	9.86		74.1
mean post tx	51.37	12.08	50.87	18.40	135.90	38.16	6.27	25.83	14.26	17.66	61.22	59.01	25.96	40.26	31.83	29.30	3.39	46.02	1.20	26.21	34.8	36.7
stdev	0.62	0.03	0.59	0.08	2.13	0.15	0.03	0.26	0.04	0.15	0.56	0.98	0.15	0.23	0.81	0.17	0.50	2.87	0.17	1.73		31.8
mean post appl	21.96	4.63	0.67	23.15	4.36	225.05	0.16	13.77	18.44	2.99	23.21	25.10	26.09	1.58	1.56	26.19	0.16	#VALUE!	0.83	0.36	22.1	9.6
stdev	0.75	1.14	0.06	1.37	1.48	27.60	0.00	1.18	0.37	0.22	1.42	0.78	2.59	0.08	0.06	1.12	0.01	#VALUE!	0.02	0.01		10.9
BL - PT	34.57	37.45	147.20	23.14	156.67	62.34	12.13	61.70	25.67	22.05	73.76	32.15	42.05	25.22	48.31	18.05	20.20	140.94	16.09	22.11		48.1
BL - PA	63.98	44.90	197.40	18.39	288.21	-124.55	18.24	73.76	21.49	36.72	111.76	66.06	41.92	63.90	78.57	21.16	23.43	#VALUE!	16.46	47.96		74.7
	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	Sample 22	Sample 24	Sample 26	Sample 28	Sample 30	Sample 32	Sample 34	Sample 36	Sample 38	Sample 40	mean values	mean values with exclusions
mean baseline	110.06	37.10	116.56	76.42	34.66	222.44	300.16	176.61	117.55	51.58	96.36	52.62	39.37	4.14	43.35	327.26	56.76	183.27	235.35	23.50	115.3	123.8
stdev	1.50	0.44	0.95	1.22	0.29	13.97	32.17	43.11	25.94	1.02	1.68	0.54	0.31	0.63	4.01	19.97	2.53	10.47	14.73	1.55		96.4
mean post tx	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	99.2
stdev	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		6.3
mean post appl	38.57	62.42	1.43	7.73	16.21	21.58	243.55	181.33	15.95	4.13	23.65	40.93	195.42	#VALUE!	2.76	73.35	2.22	2.03	2.38	2.63	49.4	40.0
stdev	1.85	7.95	0.11	0.30	1.61	1.25	57.76	4.50	0.84	1.35	3.47	2.23	33.11	#VALUE!	0.03	11.05	0.36	0.08	0.70	0.06		68.5
BL - PA	71.50	-25.32	115.14	68.68	18.44	200.85	56.61	-4.71	101.60	47.46	72.71	11.69	-156.05	#VALUE!	40.59	253.91	54.55	181.24	232.97	20.88		90.8

Appendix IV % Flow Reduction from Baseline

	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	Sample 13	Sample 15	Sample 17	Sample 19	Sample 21	Sample 23	Sample 25	Sample 27	Sample 29	Sample 31	Sample 33	Sample 35	Sample 37	Sample 39	mean values	median values with exclusions
mean baseline	2.31	-0.66	1.33		6.02		-0.40	-1.14		-2.70	0.29	1.41	-1.00	-0.49	2.39	-1.49	2.64	-0.38	4.28	5.52	1.1	0.3
stdev	3.35	5.54	1.85		10.63		0.93	0.88		2.12	1.20	1.61	1.33	0.64	2.82	1.90	6.06	6.25	9.29	19.27		2.5
mean post tx	41.60	75.45	74.66		56.35		65.81	70.15		54.33	54.78	36.18	61.45	38.21	61.23	37.20	86.02	75.29	93.36	48.75	60.6	61.2
stdev	0.70	0.05	0.30		0.68		0.15	0.30		0.38	0.41	1.07	0.22	0.36	0.98	0.36	2.07	1.54	0.95	3.38		17.2
mean post appl	75.03	90.60	99.66		98.60		99.10	84.09		92.27	82.85	72.86	61.25	97.57	98.10	43.86	99.33		95.41	99.30	86.9	93.8
stdev	0.85	2.32	0.03		0.48		0.02	1.37		0.57	1.05	0.85	3.84	0.12	0.08	2.40	0.02		0.08	0.01		16.3
BL - PT	-39.29	-76.11	-73.33		-50.33		-66.21	-71.29		-57.03	-54.49	-34.77	-62.45	-38.70	-58.84	-38.69	-83.38		-89.08	-43.24		-58.6
BL - PA	-72.72	-91.25	-98.34		-92.58		-99.50	-85.23		-94.97	-82.57	-71.45	-62.25	-98.06	-95.71	-45.35	-96.69		-91.13	-93.78		-85.7

	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	Sample 22	Sample 24	Sample 26	Sample 28	Sample 30	Sample 32	Sample 34	Sample 36	Sample 38	Sample 40	mean values	median with exclusions
mean baseline	-0.39		-0.36	1.26	0.40	3.71	-16.96	-1.54	-9.30	-0.31	0.43	-0.13		12.52	4.73	6.02	1.24	3.47	8.10	2.24		0.8
stdev	1.37		0.81	1.57	0.83	6.05	12.53	24.79	24.12	1.98	1.74	1.03		13.25	8.82	5.73	4.40	5.51	5.75	6.44		6.3
mean post appl	64.82		98.77	90.01	53.40	90.66	5.10	-4.25	85.17	91.98	75.56	22.12		#DIV/0!	93.94	78.93	96.14	98.93	99.07	89.07		89.1
stdev	1.69		0.09	0.39	4.62	0.54	22.51	2.59	0.78	2.63	3.59	4.24		#DIV/0!	0.07	3.17	0.62	0.04	0.27	0.25		33.6
BL - PA	-65.21		-99.13	-88.75	-53.01	-86.95	-22.06	2.71	-94.47	-92.29	-75.13	-22.25		#DIV/0!	-89.21	-72.92	-94.90	-95.46	-90.98	-86.83		-72.2

	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	Sample 13	Sample 15	Sample 17	Sample 19	Sample 21	Sample 23	Sample 25	Sample 27	Sample 29	Sample 31	Sample 33	Sample 35	Sample 37	Sample 39	mean values	mean values with exclusions
Baseline 1	0.0	6.9	3.7		21.8		0.0	-1.1		-5.1	1.0	0.8	-1.5	-1.3	0.0	-0.6	-4.5	-9.3	-6.5	-19.0	-0.9	-0.9
Baseline 2	0.0	0.0	0.0		0.0		0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Baseline 3	2.1	-4.0	-0.2		2.8		0.2	-2.1		-2.3	1.4	1.2	0.2	-0.7	3.9	-1.1	5.9	3.1	9.3	16.7	2.1	2.1
Baseline 4	7.1	-5.5	1.8		-0.6		-1.8	-1.3		-3.4	-1.3	3.7	-2.6	0.0	5.6	-4.3	9.1	4.7	14.3	24.4	2.9	2.9
Post Treatment 1	41.72	75.42	74.98		56.39		65.97	69.96		54.02	55.33	37.63	61.19	38.36	60.16	37.72	82.96	73.36	92.54	44.40	60.1	60.1
Post Treatment 2	42.01	75.45	74.68		56.52		65.78	70.13		54.81	54.85	36.05	61.35	38.60	60.74	37.03	86.54	74.97	92.69	48.93	60.7	60.7
Post Treatment 3	40.6	75.4	74.7		57.1		65.6	70.6		54.5	54.4	36.0	61.6	37.8	61.6	37.2	87.1	75.8	93.6	49.0	60.7	60.7
Post Treatment 4	42.1	75.5	74.3		55.4		65.9	69.9		54.1	54.5	35.1	61.7	38.1	62.4	36.9	87.5	77.0	94.6	52.7	61.0	61.0
Post Appliance 1	74.3	87.7	99.6		98.0		99.1	85.4		91.5	82.7	72.1	66.3	97.5	98.1	47.1	99.3		95.4	99.3	87.1	87.1
Post Appliance 2	74.5	90.1	99.7		98.5		99.1	84.9		92.5	84.2	72.9	61.9	97.7	98.2	44.0	99.3		95.3	99.3	87.0	87.0
Post Appliance 3	76.2	91.4	99.7		98.9		99.1	83.6		92.8	81.7	72.4	57.4	97.6	98.1	41.5	99.4		95.4	99.3	86.5	86.5
Post Appliance 4	75.1	93.3	99.7		99.0		99.1	82.4		92.4	82.8	74.0	59.4	97.4	98.0	42.9	99.3		95.5	99.3	86.9	86.9

	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	Sample 22	Sample 24	Sample 26	Sample 28	Sample 30	Sample 32	Sample 34	Sample 36	Sample 38	Sample 40	mean values	mean values with exclusions
Baseline 1	-2.0		-1.2	3.4	1.5	-1.4	-30.2	26.4	-45.2	2.3	0.1	-0.5		4.5	-5.0	2.7	-4.0	-0.8	8.2	-5.5	-2.6	-2.6
Baseline 2	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Baseline 3	-0.7		0.6	1.4	0.5	12.1	-19.2	-34.0	1.3	-1.3	-1.2	-1.2		16.0	9.6	12.7	2.6	3.4	11.3	4.9	1.0	1.0
Baseline 4	1.2		-0.8	0.2	-0.4	4.1	-18.4	1.4	6.8	-2.3	2.9	1.2		29.6	14.4	8.6	6.4	11.3	13.0	9.5	4.9	4.9
Post Treatment 1	#VALUE!		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!			#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
Post Treatment 2	#VALUE!		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!			#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
Post Treatment 3	#VALUE!		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!			#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
Post Treatment 4	#VALUE!		#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!			#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
Post Appliance 1	64.9		98.7	89.6	51.0	90.0	-27.6	-2.7	84.3	88.9	70.8	22.8			93.9	74.4	96.8	99.0	99.4	89.0	69.6	69.6
Post Appliance 2	64.5		98.8	89.9	50.3	90.5	17.8	-6.7	84.7	90.8	74.7	20.8			94.0	80.4	96.4	99.0	99.2	89.4	72.6	72.6
Post Appliance 3	62.9		98.9	90.5	52.1	91.0	21.8	-6.2	86.0	93.5	78.1	17.3			93.9	79.2	96.1	98.9	98.9	88.8	73.0	73.0
Post Appliance 4	67.0		98.7	90.0	60.2	91.2	8.4	-1.5	85.7	94.7	78.6	27.5			93.9	81.7	95.3	98.9	98.8	89.1	74.0	74.0

	Sample 1	Sample 3	Sample 5	Sample 7	Sample 9	Sample 11	Sample 13	Sample 15	Sample 17	Sample 19	Sample 21	Sample 23	Sample 25	Sample 27	Sample 29	Sample 31	Sample 33	Sample 35	Sample 37	Sample 39	mean values	median values with exclusions
mean baseline	2.31	-0.66	1.33	#DIV/0!	6.02	#DIV/0!	-0.40	-1.14	#DIV/0!	-2.70	0.29	1.41	-1.00	-0.49	2.39	-1.49	2.64	-0.38	4.28	5.52	#DIV/0!	0.3
stdev	3.35	5.54	1.85	#DIV/0!	10.63	#DIV/0!	0.93	0.88	#DIV/0!	2.12	1.20	1.61	1.33	0.64	2.82	1.90	6.06	6.25	9.29	19.27		2.5
mean post tx	41.60	75.45	74.66	#DIV/0!	56.35	#DIV/0!	65.81	70.15	#DIV/0!	54.33	54.78	36.18	61.45	38.21	61.23	37.20	86.02	75.29	93.36	48.75	#DIV/0!	61.2
stdev	0.70	0.05	0.30	#DIV/0!	0.68	#DIV/0!	0.15	0.30	#DIV/0!	0.38	0.41	1.07	0.22	0.36	0.98	0.36	2.07	1.54	0.95	3.38		17.2
mean post appl	75.03	90.60	99.66	#DIV/0!	98.60	#DIV/0!	99.10	84.09	#DIV/0!	92.27	82.85	72.86	61.25	97.57	98.10	43.86	99.33	#DIV/0!	95.41	99.30	#DIV/0!	93.8
stdev	0.85	2.32	0.03	#DIV/0!	0.48	#DIV/0!	0.02	1.37	#DIV/0!	0.57	1.05	0.85	3.84	0.12	0.08	2.40	0.02	#DIV/0!	0.08	0.01		16.3
BL - PT	-39.29	-76.11	-73.33	#DIV/0!	-50.33	#DIV/0!	-66.21	-71.29	#DIV/0!	-57.03	-54.49	-34.77	-62.45	-38.70	-58.84	-38.69	-83.38	-75.67	-89.08	-43.24		-58.6
BL - PA	-72.72	-91.25	-98.34	#DIV/0!	-92.58	#DIV/0!	-99.50	-85.23	#DIV/0!	-94.97	-82.57	-71.45	-62.25	-98.06	-95.71	-45.35	-96.69	#DIV/0!	-91.13	-93.78		-85.7

	Sample 2	Sample 4	Sample 6	Sample 8	Sample 10	Sample 12	Sample 14	Sample 16	Sample 18	Sample 20	Sample 22	Sample 24	Sample 26	Sample 28	Sample 30	Sample 32	Sample 34	Sample 36	Sample 38	Sample 40	mean values	median values with exclusions
mean baseline	-0.39	#DIV/0!	-0.36	1.26	0.40	3.71	-16.96	-1.54	-9.30	-0.31	0.43	-0.13	#DIV/0!	12.52	4.73	6.02	1.24	3.47	8.10	2.24	#DIV/0!	0.8
stdev	1.37	#DIV/0!	0.81	1.57	0.83	6.05	12.53	24.79	24.12	1.98	1.74	1.03	#DIV/0!	13.25	8.82	5.73	4.40	5.51	5.75	6.44		6.3
mean post tx	#VALUE!	#DIV/0!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#DIV/0!	#DIV/0!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	99.2
stdev	#VALUE!	#DIV/0!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#DIV/0!	#DIV/0!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!		6.3
mean post appl	64.82	#DIV/0!	98.77	90.01	53.40	90.66	5.10	-4.25	85.17	91.98	75.56	22.12	#DIV/0!	#DIV/0!	93.94	78.93	96.14	98.93	99.07	89.07	#DIV/0!	89.1
stdev	1.69	#DIV/0!	0.09	0.39	4.62	0.54	22.51	2.59	0.78	2.63	3.59	4.24	#DIV/0!	#DIV/0!	0.07	3.17	0.62	0.04	0.27	0.25		33.6
BL - PA	-65.21	#DIV/0!	-99.13	-88.75	-53.01	-86.95	-22.06	2.71	-94.47	-92.29	-75.13	-22.25	#DIV/0!	#DIV/0!	-89.21	-72.92	-94.90	-95.46	-90.98	-86.83		-72.2